THE SUB-LETHAL EFFECTS OF AMMONIUM NITRATE FERTILISER ON THE COMMON FROG *'Rana temporaria'*.

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ABSTRACT

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Increased use of fertilisers and pesticides has raised levels of agricultural pollution in surface and ground waters. Organism using these water sources are at risk of exposure to ammonium nitrate fertiliser. The effects of ammonium nitrate on spawn, larva and adult common frogs was investigated using ammonium nitrate fertiliser in solution and granular form at various stages of frog development.

The concentrations used, up to 100mg/L NO^-_3-N used reflect nitrate concentrations on agricultural land in the United Kingdom in water bodies located within and adjacent to agricultural land at times when common frogs are actively breeding or developing.

Nitrate concentrations in frog breeding ponds were high (>100mg/L NO^-_3-N) during the frog breeding season, especially when water entered from field drains, but significantly lower (<25 mg/L) for the remainder of the year. Frogs did not show a selective preference for ponds with low nitrate concentrations.

Frog spawn swelled when exposed to ammonium nitrate and its viability was reduced (87% survival in controls; 63% at 80 mg/L $NH^+_4NO^-_3$.

The 96 hour LC_{50} for frog larvae was 781 mg/L (95% confidence intervals of 587 to 942) and the 48 hour EC_{50} was 399 mg/L (95% CI = 234 to 546). Long term exposure to 100 mg/L $NH^+_4NO^-_3$ in a flow through system reduced larval survival from 85% (controls) to 53% after 96 days, with most mortality occurring during the three weeks prior to metamorphosis. Larval growth was affected marginally but with some evidence of enhanced mass in the treated larvae, especially at the lowest nitrate concentration of 25 mg/L $NH^+_4NO^-_3$. Metamorphosis in this group was earlier than in the other groups; by day 80, 48% of the metamorphs had emerged, by comparison with 38, 34 and 24% for the controls and those exposed to 50 and 100 mg/L $NH^+_4NO^-_3$ respectively. Furthermore, the mass of emergent metamorphs in the 25 mg/L treatment group was significantly higher than that of the controls.

There were higher proportions of visible abnormalities in both eggs and larvae with increased nitrate concentration (ruptured egg sacs at 40 and 80 mg/L, and 9, 19, 28 and 43% abnormalities at 0, 25, 50 and 100 mg/L $NH^{+}_{4}NO^{-}_{3}$ respectively for larvae). The severity of the abnormality increased with concentration, for example from mild tail deformity at lower concentrations to evisceration of the abdominal contents in extreme cases at 100 mg/L $NH^{+}_{4}NO^{-}_{3}$.

In the laboratory and in the field, the terrestrial activity of adults increased significantly with increased granular nitrate densities, but individuals were less likely to jump efficiently. They avoided surfaces treated with ammonium nitrate granules.

Adult laboratory frogs maintained under increasing concentrations of ammonium nitrate fed less than controls but body mass and individual oxygen consumption, although tending to be lower, were not affected significantly during the five weeks of the experiment.

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CHAPTER ONE

INTRODUCTION

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1.1 BACKGROUND

Modern agricultural practice relies on the deliberate modification of ecosystems in order to improve crop or livestock yields for human consumption and profit. Developing farming practice, such as the shifting of fertiliser application times (e.g. autumnal to vernal application for some cereals and oilseed rape) along with improved farm technologies, has led to reduced usage of compound fertilisers with an increase in the use of straight nitrogen fertilisers (ADAS 1992). As the main fertiliser application times are coincident with amphibian breeding seasons in Britain, this has caused some concern as to its effects on indigenous amphibian populations (Oldham *et al.* 1997). However, only a limited amount of research exists on the impacts of nitrates on their life history.

Since the late 1960s, amphibian decline resulting from the use of agricultural chemicals close to water bodies has caused increasing concern. They form a pivotal component to community structure, existing high within the food chain, and in some ecosystems have a biomass that can far exceed the biomass of all other vertebrates present (Roberts 1982). Few vertebrates are as dependent on environmental moisture as are the amphibians. The distribution, ecology, and, life history of a common frog and other amphibians are influenced by the distribution and abundance of water. In northern temperate latitudes, the annual breeding migration of anurans and urodeles to traditionally vernal ponds has close associations with rising temperatures and increasing levels of moisture.

Many amphibians migrate to aquatic breeding sites. Female oviparous adults deposit eggs into water, which are then fertilised externally by the male. This marks the start of the aquatic phase in the amphibian life history. For most amphibians, if the eggs are viable and fertilisation is successful, the eggs develop at a rate dependent on environmental factors especially temperature. Embryos

develop and hatch into free swimming larvae. Here the developing individuals are major consumers within the aquatic environment feeding on algae and detritus in the littoral zones of the pond. Following a growth period, larvae undergo metamorphosis whereby their bodies undergo the transition that will allow them to move from the aquatic to the terrestrial phase of their life histories. Juveniles continue to feed and grow until they become sexually mature at which point they return to the aquatic environment as mature adults to breed, thereby completing the life cycle.

The British Isles have six indigenous species of amphibian: common frog *Rana temporaria*, the common toad; *Bufo bufo*, the natterjack toad; *Bufo calamita*, the common newt; *Triturus vulgaris*, the great crested newt; *Triturus cristatus* and the palmate newt; *Triturus helveticus*.

The Common Frog (*Rana temporaria temporaria L;* referred to hereafter as *Rana temporaria*) belongs to the order of amphibians known as the Anurans. These are the 'jumping' amphibians with elongated hind limbs and without a tail in their adult life phase. Common frogs have a widespread distribution (Savage 1951) occurring in most terrestrial and freshwater habitats throughout Northern Europe. During the vernal period, adults will awake from winter hibernation and migrate to suitable breeding sites. Migratory times are dependent on factors such as geographical location and environmental cues such as ambient temperature and photoperiod.

1.2 EXPOSURE RISKS TO AMMONIUM NITRATE.

As a water-soluble compound, ammonium nitrate may pose an exposure risk to common frogs by being able to pass freely through the skin or integument. Common frogs are reliant on levels of ambient moisture in order to maintain internal moisture levels. The skin plays a fundamental role in facilitating the transport of water and gases essential in respiratory metabolism. Essential ions exchange across the integument via osmoregulation.

Terrestrial sites for common frogs usually exist close to natal sites, consisting of, for example, broadleaf woodland or a hedgerow habitat supplying adequate refuges and sufficient moisture. Urban and rural gardens with garden ponds have provided good habitats for large number of populations of common frogs and toads. The distance between their natal site and the furthest point to which they will travel in their terrestrial habitat are known as their home range. The sizes of the home range may be affected by adjacent landuse (Latham 1997).

In mainland Britain, due to the extent of intensive agricultural practices, many home ranges include an area of intense agriculture, such as a crop field or a pasture. In these areas, there is a possible risk of amphibian populations being affected by early spring applications of fertilisers. A garden population of amphibians could be affected by seasonal applications of fertilisers and pesticides for horticultural purposes.

Mitigating circumstances reduce the impact of fertiliser application. Surface topography of fields, across which individual adult frogs migrate, are not homogenous. A ploughed field in early spring is very uneven with large lumpy agglomerations of small and large soil particles. In addition, crevices and cracks together reduce the distribution of fertiliser granules on the soil surface. Many granules fall into cracks and crevices on the surface reducing the amount of fertiliser that could pose a risk to a common frog as it migrates over the field. It has been determined (Hill 1997) that approximately 40% of the fertiliser applied to a ploughed field will be lost from the surface and would therefore be unavailable for direct contact by a migrating frog. Secondly, the majority of fertilisers are applied during daylight hours, with the majority of adult frog migratory activity occurring nocturnally. Due to high water solubility, ammonium nitrate granules when applied to soil with *ca.*40 percent moisture, under constant conditions of light and temperature will dissolve in *ca.*70 minutes (Hill 1997). These three factors, topography, diurnal application and a low

environmental persistence suggest that for a nocturnally migrating frog there is a low probability of direct contact with freshly applied fertiliser at recommended field applications rates.

Although the risk to a common frog of direct exposure to ammonium nitrate appears to be low, there is still a risk of adverse effects manifesting at sub-lethal levels through the impact of ammonium nitrate solutes.

Oldham *et al.* (1997) demonstrated that the granular form of ammonium nitrate was toxic at application rates relative to the amounts applied to farmland. This was assessed by exposing individual adult frogs to granules in test tanks in the laboratory using moist blotting paper as a uniform substrate with the fertiliser granules randomly scattered across the surface at increasing exposure levels. Physiological responses were observed with respiratory efficiency of individuals being affected. The ratio of lung breaths to buccal cavity breaths was established as a physiological end-point in EC_{50} determinations (fertiliser concentration that affected 50% of a test population) Oldham *et al.* (1997). An EC_{50} of 3.6 g/sq.m $NH^{+}_{4}NO^{-}_{3}$ was established for an adult common frog. This value has a greater significance when compared to the national average application rate of 15 g/sq.m *(*ADAS 1992*).* A toxic effect was observed at a concentration five times less than the average amount applied to farmland. In the surrounding areas of Scraptoft, Leicester, the recommended field application rate is 49 g sq.m (Chas Draper, Fertiliser Manufacturers, Leicester, pers comm. 1994).

As developing embryos, common frogs are vulnerable. Embryos are surrounded in spawn, consisting of layers of gelatinous material that are permeable to water and essential electrolytes as well as allowing oxygen to diffuse to developing embryos. This acts both as a barrier from the external environment preventing desiccation and as a protective barrier from predation. Impact at the embryonic level can have serious implications for recruitment into subsequent generations.

1.3 SELECTION OF THE TEST SPECIES

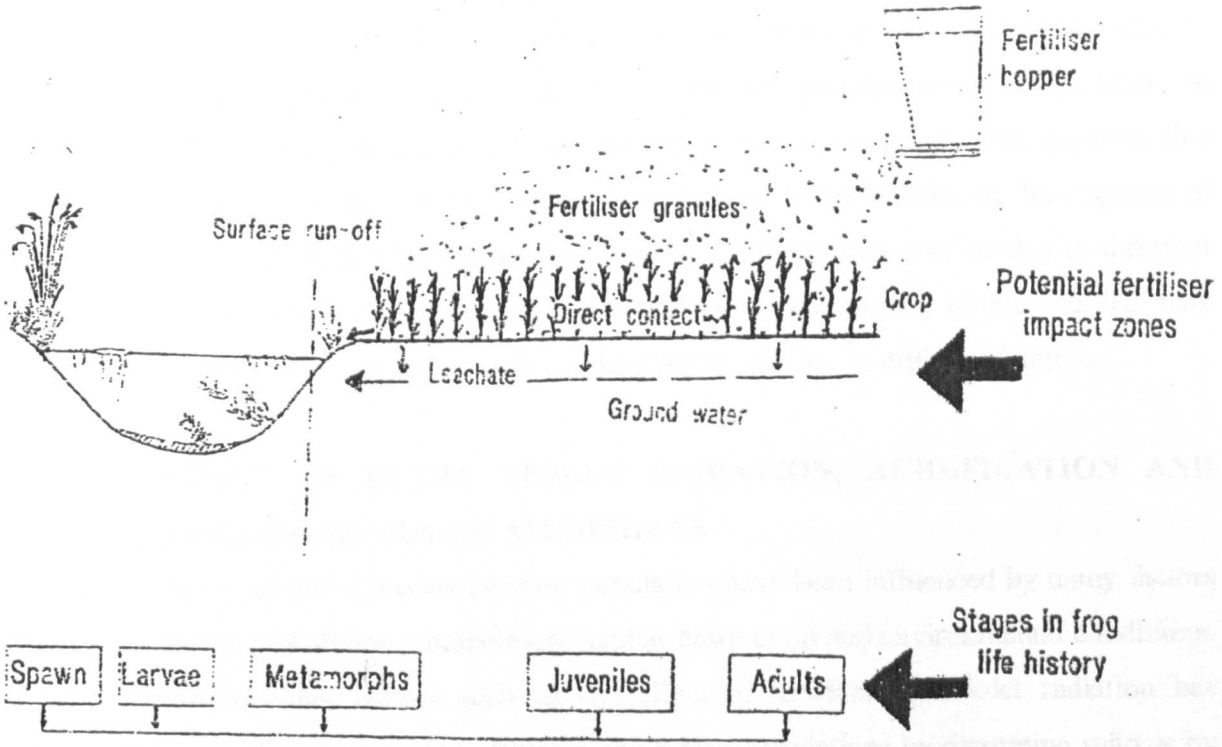
The common frog *Rana temporaria* was selected due to its recent use in experiments into the impact of fertiliser. It is a ubiquitous species on agricultural land, migrating early in the spring, and has a widespread distribution throughout the British Isles and Northern Europe. The Common frog exists in similar habitats to those of *Rana dalmatina* in which the acutely toxic effects of fertiliser were observed on *Rana dalmatina* (Wederkinch 1988). In the UK in 1990, 67% of ammonium nitrate fertiliser were applied, between March and April (ADAS 1992). This coincides with the peak period of the frog's migratory activity.

Figure 1.1 indicates the potential fertiliser impact zones following application to agricultural land adjacent to field pond. It also shows phases of the common frog life cycle that may affected.

The percentage of agricultural land treated with fertilisers in nine regions of England and Wales compared with changes in frog status within the same regions. Over the last ten years, Swan 1993 revealed a strong negative correlation (Rs = -0.89), suggesting that the application of fertiliser to agricultural land was the sole cause of the their low residence in these areas. There are other variables that may be considered such as habitat destruction and removal of suitable breeding sites following farming intensification.

Amphibian declines have been recorded in many ecosystems around the world (Blaustein 1995) and not only in isolated areas of agricultural and industrial development. There are problems in distinguishing between natural fluctuations and anthropogenic impacts. The major issue is the lack of available census data. Following a conference in Irvine, California (Pechmann 1991), three conclusions emerged concerning the decline of amphibian species. First, certain amphibian species, found in pristine habitats were disappearing at an alarming rate. Belonging to the chordata, the amphibia evolved relatively early along the evolutionary tree, and so have evolved and adapted to a wide range of varying environmental

Figure 1.1. Fertiliser impact zones and the stages of the common frog's life history at risk from exposure.



conditions. Amphibians occur within many different types of habitats. An amphibian response in one particular habitat might be detectable in species found in a tropical habitat, if the response to a chemical or physical perturbation was equally quantifiable. They may therefore be ideal as biological indicators of environmental damage. Third, there is a need to collect more data to ascertain the changes in amphibian populations, and to determine the underlying causes. Available census data is limited to localised populations of amphibians in particular geographical areas such as rain forest or moorland. This suggests that although responses may be detectable and quantifiable, the size of the response of individuals from differing habitats may be different. This may be due to different environmental conditions or due to the different evolutionary adaptations that may exist between populations of the same species existing in differing habitats.

1.4 IMPACT OF ULTRA VIOLET RADIATION, ACIDIFICATION AND AGROCHEMICALS ON AMPHIBIANS

The dynamics of a common frog population have been influenced by many factors including predation, competition, habitat destruction and environmental conditions. Environmental factors such as the effect of incident ultraviolet radiation has recently been questioned affecting amphibian populations by disrupting mitosis by actively disrupting those processes involved in the transcription and replication of DNA (Blaustein & Wake 1995). Ling (1986) revealed how acidification reduces larval development rates. Reproductive success dramatically reduces following the acidification of breeding sites (pH<4.5) (Leuven et al 1996). Acidification of pond waters leads to poor population success (Freda, 1986). Low pH affected the local distribution of amphibians in areas where acidification of water bodies was apparent (Freda & Dunson, 1986). Acute and chronic pond acidification following episodic and prolonged acidification events can promote changes in the structure of amphibian populations with poor recruitment and low levels of fecundity (Rowe, Sadinski & Dunson 1992).

The impact of pesticides on amphibians is well documented. In 1990, the United States Fish and Wildlife Service released a list of endangered and threatened species. This list included 19 taxa of amphibians. The usage of pesticides has been evolving over the last fifty years. The use of persistent pesticides such as the organochlorines to which DDT belongs, has been reduced to the use of less persistent pesticides such as the carbamates and pyrethroids (*Potter & O'Brien 1964* in Blaustein 1995). Anurans have a remarkable resistance to some of the more potent cholinesterase inhibitors. These form a major type of pesticide currently in use such as some of the carbamates and pyrethroid pesticides. The anuran resistance results from the inability of these chemicals to bind with and inhibit amphibian cholinesterase enzymes (*Potter & O'Brein, 1964* in Blaustein 1995). Many toxicity tests use lethality as an endpoint, which is questionable as behavioural effects may well manifest at concentrations many times lower than those required to achieve a kill. Teratogenic (those detrimental effects on the developing embryo that may reduce survival before hatch) effects were observed in crested newt when exposed to carbamate pesticides (Zavanella 1985). Similarly, Honrubia *et al* (1993) observed structural changes of vital organs following exposure to carbamates at non-lethal concentrations. The organophosphate pesticides are reported, to reduce the activity of skin melanophores in *Bufo melanostictus* (Pandey & Tomar 1985) making them more susceptible to predation in the field. Because of the inherent resistance to cholinesterase inhibitors, concentrations of the persistent chemicals accumulate in body fats and the liver, rising to alarming levels (Hall & Kolbe 1980). This may have subsequent effects on predators. The effects of organochlorines (such as p,p' DDT and dieldrin) at sub-lethal concentrations, (Cooke 1981) on larvae of the common frog have been highlighted for monitoring the health of effected populations in the field (biomarkers). Those pesticides considered less harmful than organochlorines, such as the pyrethroids, affect post-embryonic development (Cole & Casida, 1983).

Following research over 20 years, Berger (1987) was able to hypothesize that agricultural applications of fertilisers were responsible for declines in local

amphibian populations. The effects were most significant on *Bufo bufo, Rana esculenta, Rana aravlis* and *Rana temporaria* in addition to certain newt and salamander species. He showed that amphibian populations requiring aquatic ecosystems to reproduce, were most at risk from cultural additions. Between 1965 and 1985, Berger revealed that the number of individuals found per hectare was reduced from 300 individuals with a diversity of 12 species of frog or toad, to just 4 individuals per hectare of only 4 different species. It is important to note that Berger's conclusions were taken from census data and not from specific investigations including suitable controls and test conditions, with known chemicals. Pesticides, fertilisers, and other industrial chemicals may have been responsible for the observed affects.

Farming practices have changed, moving towards more intensive agricultural production methods (ADAS 1992). This has led to an increased fertiliser burden within agricultural landscapes. There has been a shift from the compound (nitrogen, phosphorous and potassium mixes) fertilisers, to the straight fertilisers (sodium and ammonium nitrate) fertilisers.

Baker (1993) investigated the effects of sodium nitrate on growth and survival of common toad tadpoles (*Bufo bufo)* under laboratory conditions. His research revealed that sodium nitrate in solution had clear adverse effects, leading to decreases in growth rates and higher rates of mortality. These results were inconclusive due to the possible effects of *Saprolegnia sp.* fungus on uneaten food pellets. Working on '*Littoria caerulea*', Baker (1994) modified test solutions were used with attempts to differentiate between the effects of the sodium and the nitrate ion. He revealed similar effects to those seen with the common toad such as increased mortality and reductions in growth rates. However, no distinction was made between the effect of the nitrate or the sodium ion. Hecnar (1995) investigated the impact of ammonium nitrate on amphibians. He revealed acute toxic effects of ammonium nitrate in solution on the larvae of chorus frogs (*Pseudoacris triseriata*), American toads (*Bufo americanus*), leopard frogs (*Rana*

pipiens), and green frogs (*Rana clamitans*). This was demonstrated in all test species except the green frog, following chronic exposure to sub-lethal concentrations of ammonium nitrate. In his acute studies, mortality rates were seen to increase at concentrations up to 50 mg/L NO^-_3-N as ammonium nitrate, with significant mass loss, reduced activity, and physical abnormality relative to control animals. In chronic studies, chorus and leopard frogs had significantly low survival at 10 mg/L NO^-_3-N.

1.5 AMMONIUM NITRATE AND AMPHIBIAN DECLINE

Berger (1989) attributed a decline in *Rana dalmatina* populations to elevated nitrate levels of breeding site water and its consequent effects on developing tadpoles. The species *Rana dalmatina* is a close relative of the common frog. Given the relationship it was considered possible that effects may manifest in common frogs in the United Kingdom, where application rates of nitrate based fertilisers are high relative to some countries within the European Community.

From amphibian biology, we know that the main respiratory role is played by the integument, which is also responsible for maintaining water and ionic balance directly across the skin as frogs do not have a drinking reflex (Savage 1951). Essential ions are exchanged from surrounding media via diffusion and osmosis. As ammonium nitrate is water soluble, nitrate would be present in ground water in agricultural areas where levels may be higher than background inputs. This can result in excess nitrate leaching into ponds and watercourses where amphibians breed and seek refuge. Nitrate ions in solution have the potential to disrupt the ion exchange capacity of the integument, inducing stress by direct interference of the structure of the integument. For these reasons, the integument must be considered as a potentially the most important route of exposure.

Application rates of fertiliser in the United Kingdom are more than those rates that have been observed to have an effect on adult amphibians. Straight nitrogen fertilisers account for 49% of total nitrogen inputs to agricultural systems (FMA 1997). Animal manures account for 49% and municipal wastes account for the

remaining 2%. In the United Kingdom (UK), application rates of straight nitrogen fertilisers between 1994 and 1996 when this study was conducted, ranged between 122 and 126 Kg/Ha. Total N application rates, i.e. those including compound fertilisers ranged between 147 and 152 Kg/Ha (FMA, 1997). In 1988, the average UK application rate was 148 Kg/Ha (FMA 1997), which was equivalent to a total N fertiliser consumption of *ca.* 1400 Ktonnes of nitrogen (FMA, 1997). In 1991, application rates of 169, 178 and 117 Kg/Ha respectively for arable, dairy pasture and livestock were recorded by the FMA. It may therefore be seen that fertiliser levels during the period between 1994-1996 were similar to those recorded in 1991 showing similar environmental nitrate burdens.

1.6 SUB-LETHAL EXPOSURE

The impact of chemicals on terrestrial and aquatic ecosystems may be lethal or sub-lethal, direct or indirect. Sub-lethal impairment of development affecting cell or organ morphology can reduce survival and the potential for growth and reproduction within a population. Sub-lethal effects may be manifested at a physiological level such as changes in an individual behaviour, biochemical balance, osmoregulatory ability, respiratory efficiency, locomotory ability, and reproductive ability. Many indirect effects are possible; for example, predators may be lost from an ecosystem leading to prey population explosions. Again, habitats may be destroyed or modified by algal blooming as the result of high inflows of ortho-phosphate into ponds and ditches (Young 1987).

Normal behavioural responses include seeking suitable refuge to avoid predation. If this behaviour becomes modified, it may be to the detriment of the individuals by increasing an individuals risk of predation. Perception and avoidance of pollutants is immediate and perhaps the most important behavioural response for a species exposed to habitat contamination (Werner 1996). Avoidance behaviour has been demonstrated in rainbow trout when exposed to water polluted with copper and zinc in solution, where individuals actively avoided polluted water at low concentrations (Westin, 1974). Activity levels observed in bullfrog tadpoles

showed significantly greater variability when exposed to lead nitrate at 500-1000 µg/l (Steele *et al.*, (1989). Similar variations in individual activity levels were observed in green fogs exposed to lead (Taylor *et al.*, 1990).

The avoidance of contaminated areas during reproductive periods of the amphibian's life history would selectively protect a species. This selectivity has been documented for post larval crabs preferentially settling in areas less contaminated with oil and displaying impaired locomotory activity following exposure on littoral sea shore zones. Krebs (1977). Those responses to pollutant stress relating to reproduction, migration, shelter construction and increased prey vulnerability are the most easily quantified within contaminated ecosystems and may be related to a population's functional success Butler (1978). In addition to behavioural responses, physiological responses may be quantified after an individual has been exposed to a chemical. Monitoring physiological responses would include assessment of respiratory ability, in particular levels of oxygen consumption and respiratory quotients (the ratio of carbon dioxide to oxygen in inspired and expired air). Productivity is used to quantify physiological impacts of contaminants on whole body systems. This includes food conversion efficiency, gamete production, and growth.

1.7 SUMMARY OF THESIS

The aims of this research work were to investigate the effects of ammonium nitrate on the common frog (*Rana temporaria*) at sub-lethal concentrations and under controlled experimental conditions in the laboratory and the field. This was achieved by investigating the effects of ammonium nitrate on the aquatic phases and terrestrial phases of the frog's life history the developing embryo, the tadpole and the adult.

Chapter 2 describes the nitrate issue and the potential impact to common describing the various life stages implicated and the fertiliser in question. The change in fertiliser application times and land management over recent years are

implicated in being part cause of the deleterious impacts to indigenous populations of the common frog (*Rana temporaria*).

Chapter 3 quantifies the concentrations of nitrate that exist within agricultural landscapes. The relationship between the nitrate burden of ponds and similar potential common frog breeding sites, land usage, ammonium nitrate toxicity and the migratory activity of the common frog (*Rana temporaria*) were discussed.

Chapter 4 investigates the consequences of ammonium nitrate in solution on developing frog spawns was investigated, by monitoring direct effects on spawn morphology and developing zygotes. The effects on hatching success are quantified over short-term exposure to solutions of ammonium nitrate at different concentrations.

Chapter 5 determines the acute toxicity of ammonium nitrate on frog larvae via an LC_{50} and EC_{50} tests.

Chapter 6 determines the chronic consequences of ammonium nitrate exposure on the development and productivity of frog larvae under controlled laboratory conditions at similar nitrate concentrations to those found in ponds within agricultural landscapes.

Chapter 7 investigates the impact of ammonium nitrate on adult common frogs. This was demonstrated by quantifying avoidance behaviour and individual levels of activity, during a long-term exposure trial to ammonium nitrate under laboratory conditions. Individual adults were contained in enclosed plots of a winter wheat crop under field conditions, mimicked those effects observed in the laboratory, demonstrating that the results may be extrapolated to the field.

Chapter 8 follows the development of techniques designed to investigate the impact of ammonium nitrate fertiliser on individual adult common frogs'

productivity; food conversion efficiency and respiratory ability were revealed. These were to be combined into a 'Scope for Growth' assay which could be carried out in the laboratory. The potentials of using these techniques in the field are discussed.

Chapter 9 is the discussion of the thesis. In this Chapter, the findings from all chapters will be discussed and use to assess the effects of ammonium nitrate on common frogs and highlight the risk of ammonium nitrate in the environment to the success of common frog populations.

CHAPTER TWO

AGRICULTURAL NITRATE

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2.1 COMMON AGRICULTURAL POLICY

Agriculture lends farmers the ability to attain a maximum sustainable yield of produce year in and year out, whilst maintaining low production costs to achieve acceptable profit margins. To aid farmers in this, certain articles in the treaty of Rome signed in March 1957 pertain towards a five-fold set of objectives for farmers. Articles 38-47 are those covering the Common Agricultural Policy (CAP). Firstly, to increase agricultural productivity through the promotion of technological progress and the efficient use of production factors, in particular, labour. Secondly, to maintain standards of living within agricultural communities, by increasing individual earnings potential of persons engaged in agriculture. Thirdly, attempt to stabilise markets. Fourthly, to ensure the availability of supplies, and finally, to ensure that supplies reach consumers at reasonable prices.

Common agricultural policy was a good idea at the time, introducing farmers to a "level playing field" of trade, but with its implementation came problems. These included the amount of lost revenue to member states for the produce surpluses and the disagreements between member states over produce import and export costs. Beneficiaries of the CAP have been those member states with the greatest percentages of good quality land, and not the farmers in the poorer areas. Given the technological advances in farming, rural labour forces were in decline. The European pricing mechanism provided support to farmers according to individual levels of production. This had the effect of maintaining high produce costs within the free market of the EC, meaning that the more a farmer can produce, the more financial support they would receive from their member state according to EC policy. Consequently, agricultural production was encouraged on land that would otherwise remain uncultivated. Removal of hedgerows, ponds and ditches from the rural landscape, wetlands being drained, the development of reclaimed moorlands and heathlands other varied habitat types are cleared and cultivated. Fertiliser and

pesticide applications rates have increased as the levels of farming intensification have increased over the past 40 years. More recently, the additions of fertilisers have reached a plateau with similar application being recorded (ca. 150Kg/Ha FMA, 1997) in 1995 as applied during the late 1980s and early 1990s. Intensive farming practice led to more intensive and extensive use of fertilisers and pesticides. Farming practice has become more intensive, degrading soil structure and lowering nutrient content. The net result is poorer soil water retention leading to a greater rate of leaching of cultural additions into watercourses and other adjacent habitats. This leads to direct and indirect effects on flora and fauna assembled in these habitats. Ultimately, the structure of the rural landscape as a wildlife habitat and refuge is becoming extremely fragile, held together by the farmer's ability to tend to the land. The effects on indigenous populations of amphibians such as the common frog are both direct from the physical disruption of individuals and indirect from the destruction of suitable habitat, breeding sites, and refuges. Due to the increased intensity of farming activity, chemical effects will manifest in the breeding sites where high burdens of agricultural pollutants.

Member states of the European Community now offer subsidies to farmers to reduce the amount of produce held as a surplus, effectively paying the farmer for not farming an area of land for a certain period. This scheme known as 'set-aside' highlights the benefits of farmland management in attracting once banished forms of wildlife back into habitats that have been 'set-a-side'; a positive move to counter intensive practices.

2.2 NITRATE IN THE ENVIRONMENT.

The nitrate ion is ubiquitous in the environment, as an integral compound of the nitrogen cycle. Nitrate is considered relatively harmless to human beings at low concentrations and is not considered toxic in surface waters (Chalmers, 1990). In 1990, concerns over increasing concentrations of nitrates in surface and ground waters increased. This increased public awareness as to the potential of nitrate

pollution in water bodies and watercourses, highlighting that agricultural application of fertilisers such as ammonium nitrate are a major source of surface water contamination (World Health Organisation, 1990).

Nitrogen exists naturally as a gas (N_2) when not fixed in the environment. As N_2, it is very stable and normally unreactive. Biological fixation allows the element to become reactive at normal temperatures. Animals excrete organic waste products containing fixed nitrogen. Bacteria and microbes digest and decompose these waste products, producing ammonia, and ammonium ions. Ammonia and ammonium specific bacteria in the soil nitrify these compounds forming nitrites (NO_2^-). Nitrifying bacteria nitrify these ions of ammonium and ammonia, forming nitrates. Nitrates are readily soluble and are actively taken up into plant tissue and utilised in production. Nitrates exist in large quantities in cereals and root crops and are absorbed or assimilated by those organisms (e.g. herbivores and omnivores) that consume the plant material.

Bacteria are also responsible for fixing nitrogen sources (waste products) from microbes and plants, especially during the decomposition of organic wastes. Inevitably, as the nitrate ion has a high solubility in water and it has a high mobility within the environment. Due to high mobility of nitrates in surface waters, they pose a potential risk to flora and fauna present in these habitats. The implications to common frogs are twofold: firstly, they have a permeable skin, which is a potential route of entry for the soluble nitrate ion. Secondly, they have a dependence on aquatic habitats for reproduction, larval development, foraging, and hibernation.

Nitrates are a precursor of a proven toxin, nitrite, causing a condition known as methaemoglobulinanemia, which reduces the oxygen carrying capacity of the oxygen carrying component- haemoglobin. A human baby condition is called "blue-baby syndrome". As a direct result, the World Health Organisation (1980) imposed restrictions on the amount of nitrate permissible in potable waters to a

level not exceeding 50ppm-nitrate nitrogen (equivalent to approximately 11.3-ppm nitrate).

The accumulation of high nitrate occurs in surface waters in areas where intensive agriculture persists with multiple applications of fertiliser made during the year. Arable areas with poor soil structure, texture, and composition have poor nitrate retention qualities. Agricultural areas with high incident annual rainfall during the early growing months of a crops development, ameliorate the nitrate burden of ground waters, leading to elevated levels of residual nitrate in adjacent surface waters.

2.3 THE NITROGEN CYCLE

As has already been mentioned, an appreciation of the nitrogen cycle is of great importance in understanding the dynamics of the natural cycling of nitrate entry and release from the ecosystem, and the processes involved in the transportation of nitrate in the field.

Gaseous Nitrogen

Nitrogen is an essential element acting as a metabolic intermediate, as an essential constituent of amino acids required in the structure of proteins in animals and plants. The major reservoir of inorganic nitrogen is gaseous N_2, which comprises 78% of the atmosphere. Microorganisms found in the lithosphere, such as blue-green algae metabolise nitrogen directly, in the synthesis of essential proteins. These may be assimilated by other organisms.

Fixed Nitrogen

There is a steady return of nitrogen to the atmosphere, as nitrates, nitrites, or ammonium ions are converted into nitrogen gas and released back to the atmosphere by denitrifying bacteria. The natural nitrogen cycle exists within a dynamic system, where the denitrification and nitrification are at equilibrium.

Agricultural Nitrogen

In agriculture, the process of nitrate cycling is complex. Soil is under stress from intensive agricultural practice affecting both the soil nitrogen reserves and physical structure. In medieval times, crops were grown in farming systems that required periods of fields being laid to fallow. This permitted the soil time to restore the levels of nitrogen. Today, the increasing demand for cereal and root crops has seen the fallow period replaced by applications of fertiliser or the use of a leguminous cover crop such as clover to replace soil nitrogen levels. The application of fertilisers is a major source of inorganic nitrogen commonly applied as nitrates, in the soil. This disrupts the equilibrium of the natural nitrogen cycle, but within intensive agricultural practices, forms an essential component of arable farming production. The use of field drainage systems with intensive farming practices leads to an increased nitrate burden in static waters in agricultural areas. This occurs through surface and ground water run-off. Agricultural use of fertilisers and pesticides has played major roles in enhancing the productivity of many farms. Fertilisers improve productivity of arable crops and pesticides remove pests both in and on the soil, and on developing crops and improve the health of cattle (ADAS, 1997).

2.4 FERTILISER USAGE IN THE UNITED KINGDOM

In the United Kingdom today, three main types of fertiliser are applied to cultivated land, namely human and/or cattle liquid manures (organic), compound fertilisers and straight fertilisers (ADAS, 1997). Production of organic fertilisers may be from plant or animal material. Organic fertilisers supply nitrogen in a non-

accessible form, which must be assimilated before it becomes available to plants. They have advantages over inorganic fertilisers, in that they are not immediately soluble in water, and therefore not readily leached from the soil. They degrade slowly, becoming available to plants over a period and may be used as a slow release fertiliser. They may be applied at relatively heavy rates, without risk of injury to roots of the germinating seed as they have little ionic activity. They promote microbial activity in the soil profile. They are however much more costly per unit of plant food and as such are not generally used in agriculture due to the cheaper costs of inorganic fertilisers. Combination fertilisers of nitrogen, phosphorous and potassium (N P K fertilisers) are known as compound fertilisers. These are less widely applied as many farmers opt for straight nitrogen based fertiliser applications. Straight fertilisers have a high percentage content of one particularly element. In most cases, this is usually nitrogen in the form of nitrate. A compound fertiliser may have an N: P: K ratio of 25: 5: 5, where as a straight fertiliser such as ammonium nitrate ($NH_4^+NO_3^-$) has an N: P: K ratio of 34.5: 0: 0, a much higher proportion of nitrogen.

Straight nitrogen fertilisers are applied to a developing crop during the early stages of the crop development and again at crucial stages. This will increase the available inorganic nitrogen reservoir in the soil, improving growth rates, and yields. It follows that profits are also improved.

Ammonium nitrate is an important fertiliser sold as a straight fertiliser or as a component in compound fertilisers. Ammonium nitrate is sold as a straight fertiliser containing approximately 33.5 – 34.5 % nitrogen: this material is very soluble in water and hygroscopic, so requires storage in dry places in sealed bags. Being soluble in water, ammonium nitrate may be used as a source of nitrogen in liquid fertilisers. It is supplied either as granules or prills (flattened disc-like granules). Rotating the fertiliser powder in rotary drums in the presence of an inert granulating and coating agent makes granules. The size of these granules is dictated by optimal farming application rates. Rapid cooling of fertiliser liquids

held at various depth forms prills. The depth of the fertiliser liquid before cooling determines the size of the prills.

Other fertilisers exist, with their use dictated by expense. These include urea, which can be synthesised artificially. This is usually applied as a liquid component of compound fertilisers. Market prices for urea are approximately 20% more than ammonium nitrate. Ammonium sulphate may also be used, but has been replaced in many areas by ammonium nitrate as it has a greater proportion of nitrogen compared with the sulphate-based fertiliser. Compounds such as calcium ammonium nitrate, anhydrous ammonia, aqueous ammonia, potassium nitrate, and sodium nitrate tend not to be used on a large scale due to their relatively high production costs. Phosphate and potassium tend to be added to liquid compound fertilisers; used as quick release fertilisers; or as insoluble rock phosphates or slags, which are applied to maintain the soil status over the long periods of time.

2.5 FERTILISER APPLICATION RATES.

Straight nitrogen fertilisers account for 49% of total nitrogen inputs to agricultural systems. Animal manures account for 49% and municipal wastes (2%) account for the remaining inputs). In the period 1989-1995, the overall average nitrogen fertiliser application rate for the British Isles was 153 kg/ha (EFMA report 1997/1998). See Table 2.1.

Table 2.1 Fertiliser use in England & Wales, 1989 –1995

Crop Type	Average Nitrogen application rates (Kg / ha)						
	1989	**1990**	**1991**	**1992**	**1993**	**1994**	**1995**
Spring wheat	157	149	144	154	144	161	141
Winter wheat	182	184	187	188	186	187	194
Spring barley	94	97	90	92	94	96	99
Winter barley	143	138	139	143	138	144	145
Maize	76	91	181	205	167	190	171
Main crop potatoes	208	193	127	122	115	127	122
Sugar beet	121	122	227	197	181	182	190
Oilseed rape	233	228	77	66	65	69	70
All tillage	163	198	166	125	130	128	127
Permanent grass	122	155	138	138	139	141	140
All crops and grass	157	159	161	149	145	150	150

Compiled from the 'British Survey of Fertiliser Practice 1989-1995

Within Europe, in 1998 the United Kingdom (ca. 1400 thousand tonnes total nitrogen) was ranked third after France (2518 thousand tonnes) and Germany (1760 thousand tonnes) in total nitrogen consumption rates.

The Ministry of Agriculture Food and Fisheries (MAFF) following a two year pilot scheme recognised that nitrate losses from agricultural land were contributing to nitrate pollution of surface and ground waters in the United Kingdom. They confirmed that losses of nitrate into surface waters were dependent on the crops grown in adjacent fields. In areas where a green cover crop such as clover or grasses were allowed to establish, nitrate losses were considerably lower (MAFF, 1993).

2.6 FERTILISER APPLICATION TIMES.

In 1990, 67% of ammonium nitrate fertiliser was applied between March and April in the United Kingdom. This compares with 35% of ammonium nitrate applied during the same period in 1980 (ADAS 1997). Vernal applications of fertiliser reduce the number or application (and amount of fertiliser) applications a farmer has to make during a year. In 1980, a winter and a spring application of fertiliser onto a single crop such as Winter Wheat were common. It reduces the amount of nitrate lost during the winter months, when incident rainfall is expected to be higher. Applications more cost efficient. Today, farmers tend to apply fertilisers in the early months of the year, once the number of day hours dry is equal to the number of day hours wet. This determination prevents drastic losses of fertiliser from over moist soils.

CHAPTER THREE

NITRATE SAMPLING PROGRAMME AND AMPHIBIAN MONITORING OF RURAL PONDS IN LEICESTERSHIRE

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NITRATE SAMPLING PROGRAMME AND AMPHIBIAN MONITORING OF RURAL PONDS IN LEICESTERSHIRE.

3.1 INTRODUCTION

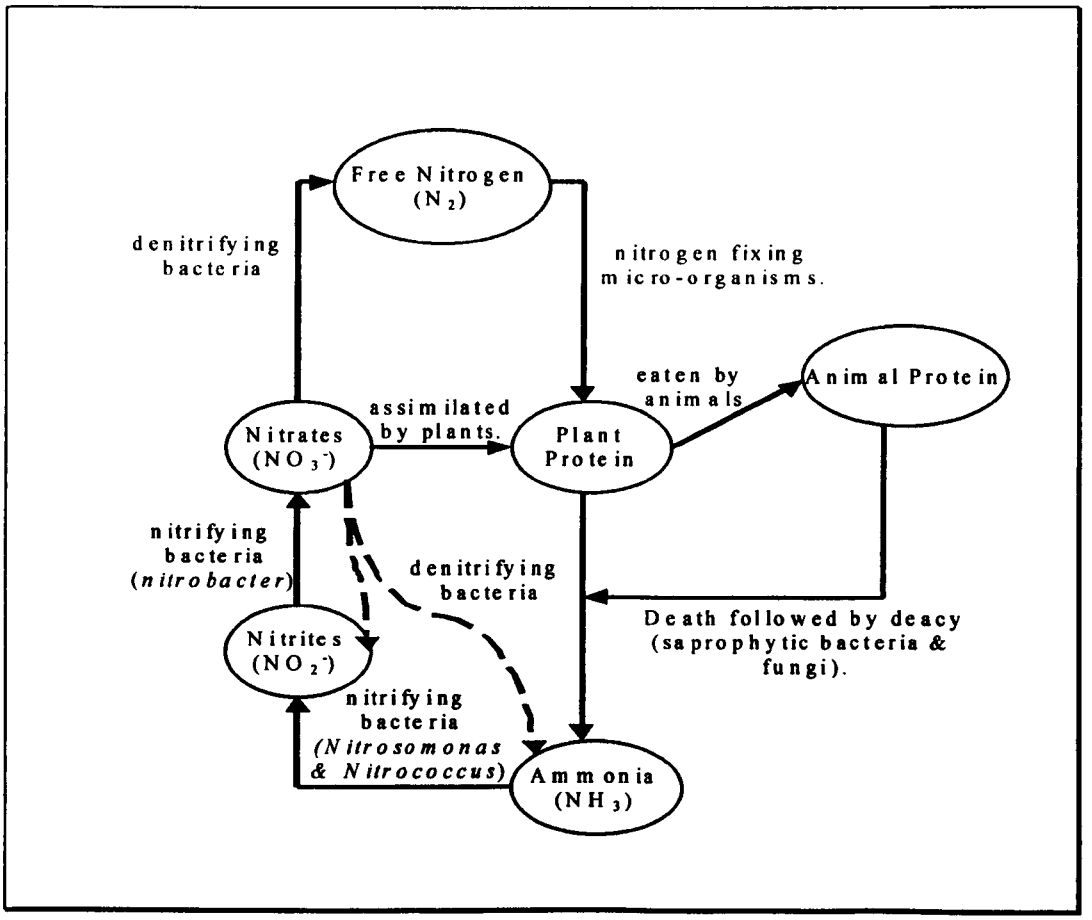
To establish the impact of nitrate on the frog, the ranges of residual nitrate concentrations were measured in selected ponds throughout the year.

The nitrogen cycle is the process which describes the cycling of nitrogen in various forms throughout the environment. Following the application of ammonium nitrate to a field, the breakdown products of ammonium ions are denitrified through the action of nitrite bacteria converting them to nitrites. Nitrate bacteria convert nitrite to nitrate, then available for uptake by plant roots (Garrels 1975). The nitrogen cycle is illustrated Fig 3.1.

The concentrations of nitrate present in ponds within agricultural landscapes were monitored between 1994 and 1996. These data were used to set the range of concentrations used in laboratory studies and facilitated rangefinding experiments. Additional information on how each site was fed, either by field drains or through surface runoff, was collated to establish whether the feed source to each pond dictated the range of nitrate concentrations to which it was exposed.

Each site was monitored for the presence of amphibia to establish whether they showed a preference for ponds with high or low of nitrate levels.

Figure 3.1. The nitrogen cycle, summarising the cycling of nitrogen in the environment (Garrels 1975).



3.2 METHODS

A three-year programme (1994-1996) of pond monitoring was initiated on the 11 February 1994, and divided into two phases. The first phase in 1994 was used to establish the levels of nitrate in six ponds and a ditch that were selected due to their proximity to the campus and due to the diverse agricultural lands adjacent to each of the ponds. These ponds were monitored over a twelve-month period. This would give an indication of the yearly variations in residual nitrate concentration of rural ponds and indicate at which time of the year common frogs were most at risk from nitrate exposure.

Amphibian monitoring was undertaken using sweep netting (all species), bottle trapping (newts), and spawn counting (frogs). Inspections of marginal water plants for newt eggs were also carried out. Once the first spawn was detected, pond observations were increased from once a fortnight to once a week. Land usage adjacent to the ponds was established to identify whether this type of land use could have affected the drainage into the pond. Where possible, the way in which each of the pond sites was fed with supply water was established.

The second phase (1994-1995) of the monitoring programme involved sampling a large number of ponds covering diverse pond sizes and locations. All sites were between eight and sixteen km from the laboratories. In the second phase, nitrate concentrations, the presence or absence of amphibians and the type of drainage into the ponds (drain or surface runoff) were established. This phase started on 6 December 1994 and finished on 22 June 1995. Fifty-three ponds were visited on a fortnightly basis, in addition to those ponds selected during the 1994 (first phase) sampling programme.

In the third phase (1996) additional sampling and amphibian monitoring was undertaken using the same 53 sites with sampling restricted to the period of frog breeding (February to April). The aim was to augment the information collected during the previous year's sampling and monitoring at the time of year when frogs were considered to be most at risk from nitrate exposure.

3.2.1 Phase One Nitrate Sampling during 1994.

In the first phase, six rural Leicestershire pond sites and a ditch were selected (Table 3.1 and Appendix 1 & 2). Five sites were located in the Scraptoft area, with the remainder of sites located near Melton Mowbray, Leicestershire. At two of the sites, it was possible to identify the inlets (land drains) and outlets. Here, nitrate measurements were taken at the inlet, outlet and from the pond edge at a point equidistant between the two, known as the central point. At the remaining sites, a single sampling point was selected along the bank of the pond.

Table 3.1 Pond sites monitored during Phase One sampling in 1994.

Site name	Sample points*	Grid reference	Approximate surface area (sq.m)	Adjacent land use.	Species of amphibians observed
Wood Pond (Plate 3.1)	1	SK 665053	15	Mixed broad leaf woodland and unimproved pasture.	Rana temporaria Triturus vulgaris
Fish Pond (Plate 3.2)	3	SK 661051	9	Winter wheat and sheep pasture.	Triturus vulgaris
Nature reserve pond (Plate 3.3)	1	SK 661053	8	Winter wheat and horse pasture.	Rana temporaria Triturus vulgaris
Keyham Pond. (Plate 3.4)	1	SK 663058	9	Winter wheat. Maize	Rana temporaria Triturus vulgaris
Keyham Ditch. (Plate 3.4)	1	SK 663058	1	Winter wheat.	Rana temporaria
Far Corner Pond. (Plates 3.5-3.7)	3	SK 676148	90	Winter wheat.	Rana temporaria Triturus vulgaris Triturus cristatus
Spinney Pond. (Plate 3.8)	1	SK 682153	50	Mixed broadleaf woodland, rape, and maize crop.	Triturus vulgaris Triturus cristatus

Each pond site had one (marginal) sampling point, with the exception of Fish Pond and Far Corner Pond, which had three sampling points; inlet, outlet and pond edge.

Each pond site was visited weekly, with the concentrations of nitrate (mg/l NO_3^- N) being established using a hand-held, portable nitrate meter (BDH-Merck 'Reflectoquant RQ-Flex meter'). Chemically treated test strips (BDH-Merck 'Test Strips') were dipped into the pond water at the predetermined marginal sampling points at each site. Following a 60-second development time, the test strip was inserted into the meter, where the concentration of nitrate (mg/l NO_3^-N) was analysed colourimetrically using the hand held meter. Analytical accuracy of the meter was ± 0.5%, with the meter calibrated each time a new container of strips was opened, using a calibration strip provided with each new tube of strips. The nitrate determinations were usually within the range of 3 to 90 mg/l NO_3^- N. Nitrate concentrations less than 3 mg/l were displayed as 'LOW' on the digital display of the meter, with concentrations greater than 90 mg/l indicated by 'HI'. Samples with high (>90 mg/l NO_3^- N) readings were re-measured in the field using samples diluted 1:1 with deionised water. At fortnightly intervals, the meter

was calibrated using a 50mg/l ammonium nitrate solution corrected for the nitrate content to attain a nominal nitrate-nitrogen concentration of 100 mg/l NO_3^-N. At no point were the readings from the meter more than 5% greater or less than the nominal concentration of the calibration standard. Standard solutions were prepared fresh at each calibration, using ammonium nitrate granules and deionised water (1-litre).

3.2.2 Phase Two Nitrate Sampling during 1995 & 1996.

In the second year (1995), 53 ponds were visited and sampled, including the seven selected sites from 1994. Nitrate concentrations, presence, or absence of amphibians, adjacent land use and the feed source of the water in the ponds were established.

In the final year, (1996), sampling of the 53 sites sampled in 1995 was repeated during the common frog breeding season between February and April.

3.3 RESULTS

3.3.1 Nitrate Concentrations in Ponds during Phase One (1994).

Table 3.2 summarises the nitrate levels recorded at each of the sites. A more detailed description of each site may be found in the Appendix 1. Nitrate levels within the ponds are summarised in Table 3.2. High concentrations of nitrate in the ponds coincided with the first appearance of amphibians (Figure 3.2) within the ponds. Figures 3.3 to 3.5 show the nitrate levels for each of the sampled sites during 1994. At each pond, the trend during the sampling period was for high nitrate levels during the spring (February-May), early summer (June-July), and late winter (December-January) with levels decreasing in the summer and autumn (August-November). In some cases, the nitrate concentrations dropped to <3 mg/l NO_3-N.

The highest mean level of nitrate recorded at any of the sites was 75.5 mg/l NO_3-N, measured in November at Fish Pond (Table 3.2)

The lowest mean monthly nitrate levels were recorded for Wood Pond with consistently less than 3 mg/l as NO_3-N. Nature Reserve Pond also recorded very low mean monthly concentrations of nitrate for most of the year (Table 3.2).

Figure 3.2. Mean nitrate concentrations in all sampled ponds during Phase one (1994-1995), showing the approximate breeding season of the common frogs. (standard erros shown).

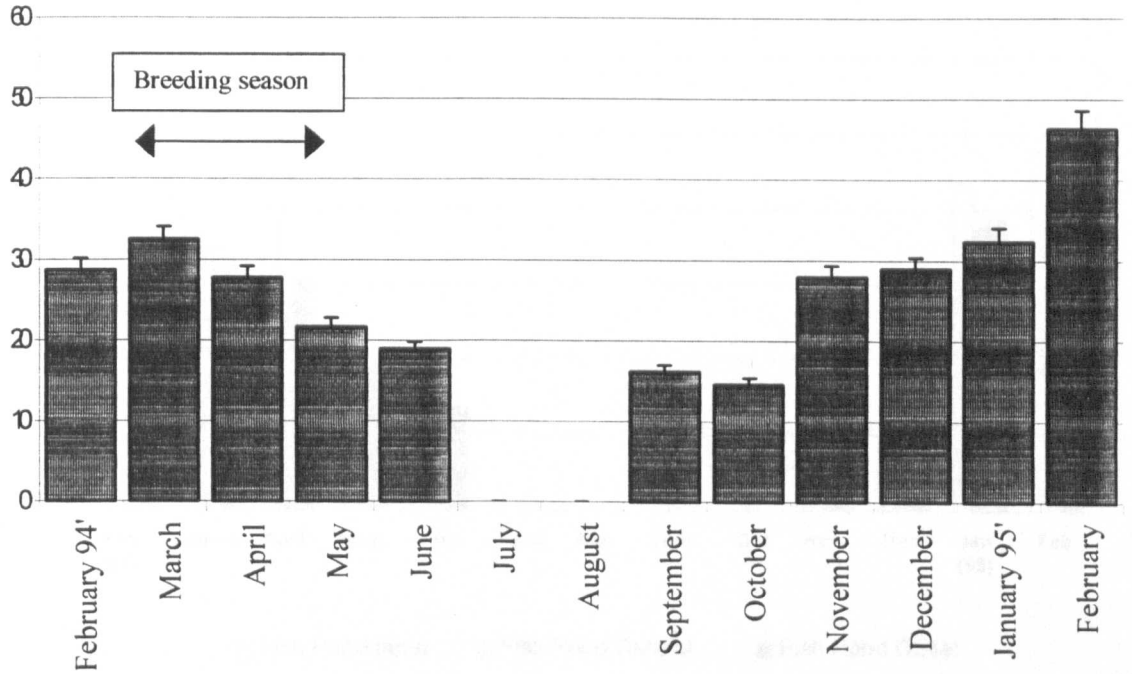


Figure 3.3. Fish Pond (SK 661051) monthly mean concentrations of residual nitrate (mg/l NO_3-N) recorded for the field drain inlet, the main body and the outlet of the pond.

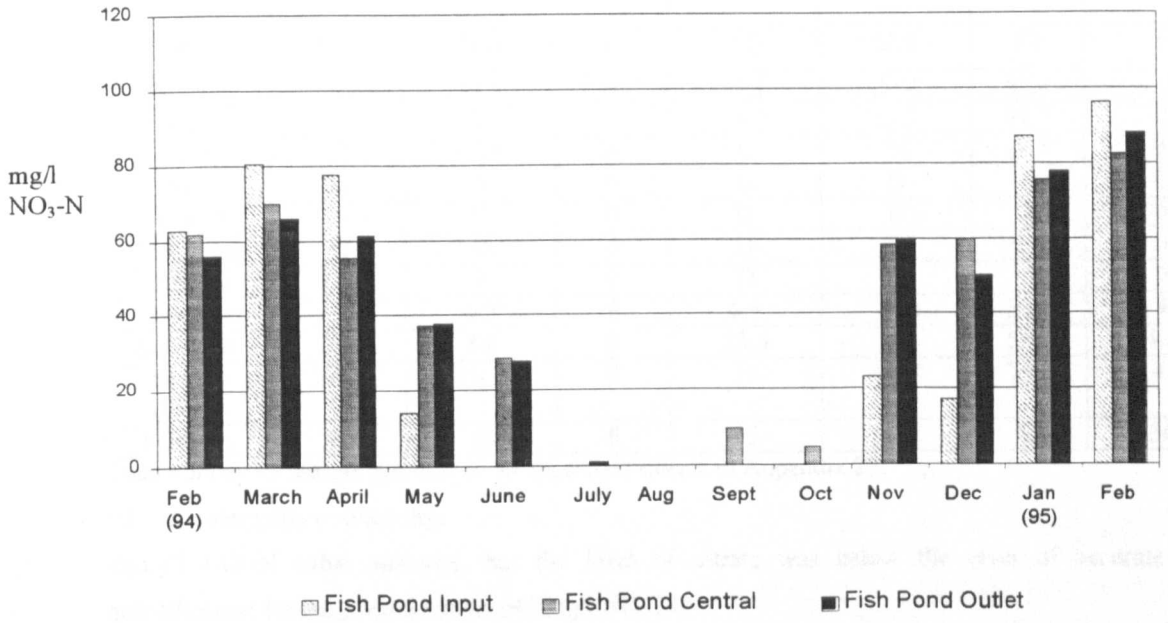


Figure 3.4 Far Corner Pond (SK 676148) mean monthly concentrations of residual nitrate (mg/l NO_3-N) recorded for a field drain inlet, the main body of the pond and the outlet of the pond.

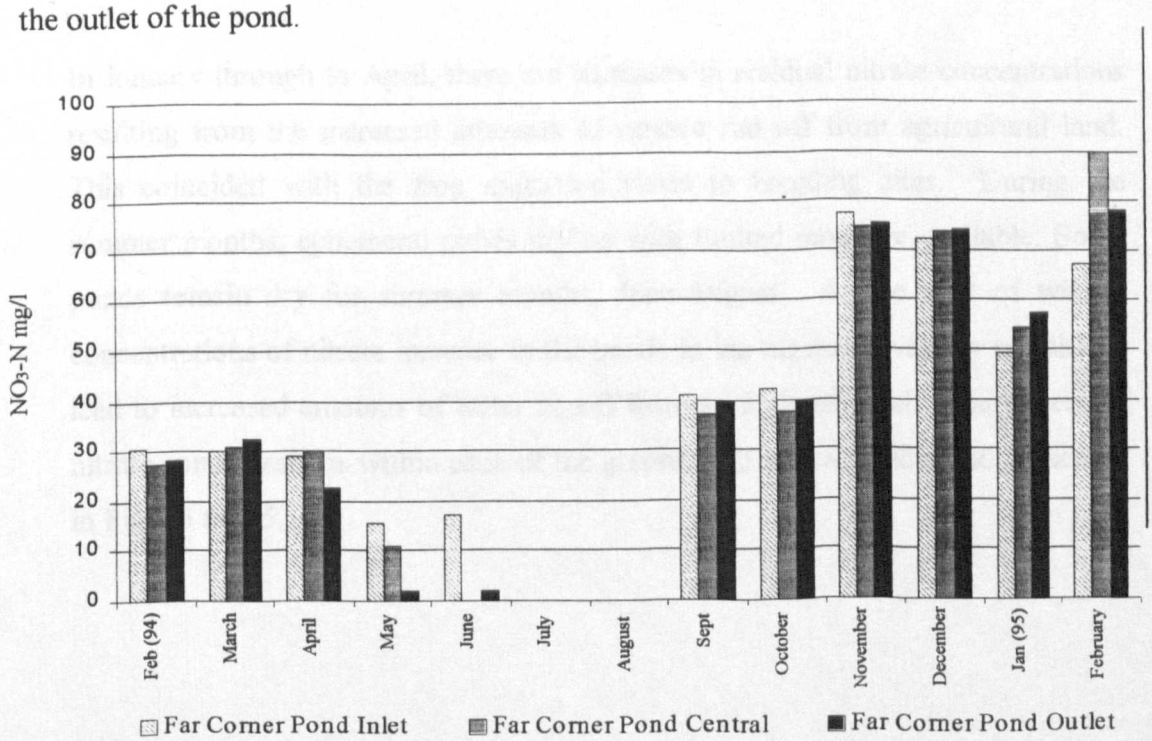


Table 3.2. Mean monthly nitrate concentrations (mg/l NO_3-N) from pond sites monitored during Phase One sampling in the 1994-1995 season.

Month	Far Corner Pond	Spinney Pond	Wood Pond	Keyham Pond	Keyham Ditch	Fish Pond	Nature Reserve Pond
February (1994)	27.5	47	0	14.7	16	61.7	5.3
March	30.8	49.4	0	17	22.8	70	5
April	30	40.5	0	18.2	20.4	55.4	2.3
May	11	23.3	0	15.3	21.3	37.5	0
June	D	17.3	0	6	24	28.5	0
July	D	0	0	D	D	0	0
August	D	0	0	D	D	0	0
September	37	0	0	D	D	9.8	0
October	37.6	0	0	D	D	5	0
November	74.8	9.2	0	6.5	12.8	58.3	9
December	73.8	8.8	0	13.3	13.6	59.3	4.8
January (1995)	54.3	19.3	0	14	16.8	75.5	14.3
February	90	45	0	15	16	82	30

Total number of sampling times; n=46 (raw data shown in Appendix 2)

'D' - denotes pond or ditch dry.

0-mg/l NO_3-N value indicates that the level of nitrate was below the level of accurate quantification for the portable meter (<3 mg/l NO_3^-N).

Spinney Field Pond recorded the second highest nitrate concentration between February and June 1994 (Table 3.2). At Fish Pond, the highest nitrate levels were recorded over the same 5 month period with a weekly average of 51.9 mg/l NO_3^- N.

In January through to April, there are increases in residual nitrate concentrations resulting from the increased amounts of surface run off from agricultural land. This coincided with the frog migration times to breeding sites. During the summer months, ephemeral ponds dry up with limited moisture available. Some ponds remain dry for summer months, June-August. At the start of winter, concentrations of nitrate increase in the ponds as the seasonal weather conditions lead to increased amounts of water runoff from agricultural land. The pattern of nitrate concentrations within each of the graphs is discussed below and presented in Fig 3.3 to 3.5.

3.3.1.1 Far Corner Field Pond (SK 676148), Brooksby, Leicestershire.

Far Corner Field Pond was fed with via field drains and surface runoff. The period from February 1994, to 1995 revealed a temporal change in nitrate concentrations. In Figure 3.4, each bar represents the mean residual nitrate concentration during each month for each of the three sampling locations within the pond. The concentrations recorded over the first three months (February to April (1994)) range between 22 and 33 mg/l NO_3^- N. During May and June, concentrations of nitrate decreased at all three sampling locations. In May, the concentrations recorded for the inlet and central sampling point were high, compared to the outlet. In July and August, the water level in the pond dropped with no recordings possible at the outlet (Table 3.2 and Appendix 2). A Winter Wheat was harvested from the surrounding land between the 22 and the 26 July 1994. In September, nitrate concentrations increased to *ca.*40 mg/l NO_3^- N. The water level increased over the winter months and was back to levels first observed in February (1994) . Nitrate concentrations were similar at the three sampling points and were maintained during October increased during November and December. In February (1995), the mean nitrate concentration at the field drain inlet increased, with concentrations in the main body of the pond and the outlet remaining similar to those recorded for November and December.

3.3.1.2 Fish Pond (SK 676148), Scraptoft Lane, Scraptoft

Fish Pond was supplied with by surface run off and via land drains. A sheep grazing pasture adjacent to the pond was applied with fertiliser on 14 February 1994. Nitrate concentrations in early February were high at all three sampling points, with mean nitrate concentrations during February, March and April being approximately

60 mg/l NO_3^- N. In the first half of the year, nitrate concentrations at the central sampling point were between 15 and 81 mg/l NO_3^- N, with the highest nitrate concentration recorded during March and the lowest during June. Nitrate concentrations at the inlet to the pond between February and March were high, between 48 and 90 mg/l NO_3^- N. Concentrations at the outlet between February

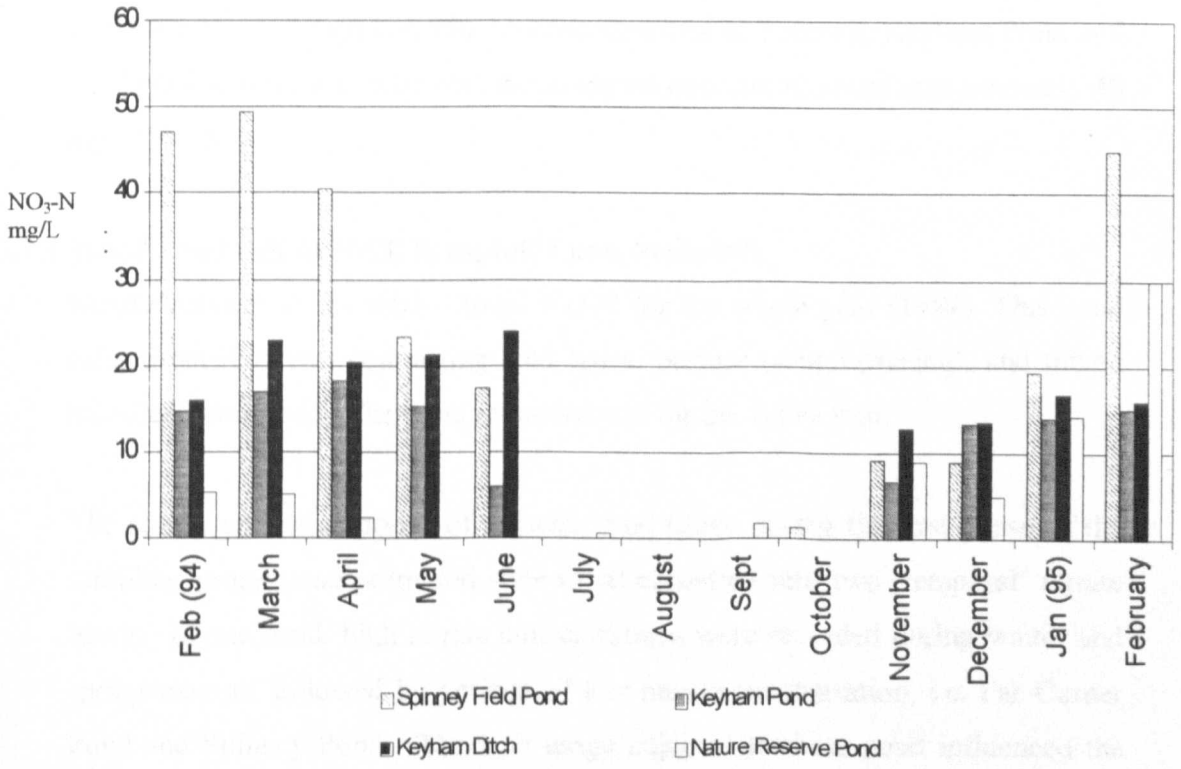
and mid-June were between 27 and 76 mg/l NO_3^-N. In July, the outlet dried up as the water level dropped in the pond. This point remained dry until November. The central sampling point recording <3 mg/l NO_3^-N on all sampling occasions over the same period. At the start of November, nitrate concentrations increased at the central and outlet sampling points. On 11 November 1994, there was a peak, with concentrations at all three sampling points greater than 90mg/l. In January and February 1995, the concentrations of nitrate at the three sampling sites were similar (approx. 80 mg/l NO_3^-N).

3.3.1.3 Spinney Pond (SK 682153), Keyham Pond and Keyham Ditch (SK 663058) and Nature Reserve Pond (SK 661053).

All four of these sites were fed by surface run off. Figure 3.5 shows the mean monthly nitrate concentrations recorded at the four sites. Nitrate concentrations recorded in Spinney Pond over the first three months of sampling in 1994 were >40 mg/l NO_3^-N. Animal manure was applied to the field (as a liqor and silage), adjacent to the pond during late January and early February 1995. During May, nitrate concentrations decreased by *ca.*20 mg/l NO_3^-N. They continued to decrease in June, with nitrate concentrations from July to October being <3 mg/l NO_3^-N. In November and December, concentrations increased with the mean monthly nitrate concentrations of 9.2 and 8.8 mg/l NO_3^-N. At the start of 1995, animal liqor and silage were applied during early February and nitrate concentrations in January and February were similar to those recorded during the same period of 1994.

Nature Reserve Pond had consistently low nitrate levels throughout 1994, with concentrations peaking at 10 mg/l NO_3^-N in March 1994. In the period between June to October, nitrate concentrations were <3 mg/l NO_3^-N. Nitrate concentrations increased towards the end of 1994 with nitrate concentrations increasing to 16 mg/l NO_3^-N in November. In 1995, concentrations during January and February increased, with mean value of 14.3 with a high of 30 mg/l NO_3^-N. Nitrate concentrations in Keyham Pond and Keyham Ditch were between

Figure 3.5 Monthly mean residual nitrate concentrations (mg/l NO_3-N) for Spinney Field Pond (SK 682153), Keyham Pond and Keyham Ditch (SK 663058) and Nature Reserve Pond (SK 661053). All four sites were examples of ponds supplied either by rainfall or surface run off and not by land drains.



14.7 and 22.8 mg/l NO_3^- N during the period between February and June 1994. Concentrations in the ditch were higher than the pond over the same period. The pond and ditch were dry between July and November. In the period between November (1994) and February (1995), nitrate concentrations were similar to those observed at the start of 1994, ranging between 7 and 16.8 mg/l NO_3^- N. Concentrations of nitrate at Keyham Pond remained high during May and June. In September and October, nitrate concentrations at Spinney, Keyham Pond and Keyham Ditch were similar with mean nitrate concentrations of approximately 40 mg/l NO_3^- N.

3.3.1.4 Wood Pond (SK 661053) Scraptoft Lane, Scraptoft.

Nitrate concentrations were <3mg/l NO-N for the whole year (1994). This pond existed between two contrasting land types: pasture (rough grazing), and mixed broadleaf woodland. The pond remained wet for the entire year.

The results and descriptions of adjacent land usage during the first phase of the sampling programme permitted sites to be classified into two 'temporal' nitrate bands. In one band, high nitrate concentrations were recorded during winter and spring months, followed by periods of low nitrate concentration, i.e. Far Corner Pond and Spinney Pond. The land usage adjacent to these pond influenced the nitrate concentrations recorded. In the second band, nitrate concentrations were consistently low for the whole year. i.e. Wood Pond and Nature Reserve Pond, where residual nitrate concentrations were less than 16 mg/l with one exception at Nature Reserve Pond on 11 November 1994, following a period of heavy rainfall. Adjacent land use and topography affected nitrate concentrations at most sites.

3.3.2 Amphibian monitoring during Phase One (1994).

Amphibians were present at all sites visited during Phase One monitoring. Common frogs and common newts were found at six of the seven sites. Common newts were not found in Keyham Ditch and common frogs were not found in Spinney Field Pond. Both of these sites were moderate nitrate sites, with February

to June nitrate concentrations between 16 and 24 mg/L $NH^{+}_{4}NO^{-}_{3}$ for Keyham Ditch and between 17.3 and 49.4 mg/L $NH^{+}_{4}NO^{-}_{3}$for Spinnet Filed Pond. Crested newts were found at two sites, namely Far Corner Field Pond and Spinney Field Pond. Frog spawns were found at five of the seven sites. The number of spawn clumps was counted at each site to assess population size. Spawn was checked for viability in situ. In all cases (where spawn was found) viable embryos were observed. Over-wintering crested newt larvae were found at two sites. Far Corner Pond recorded among the highest residual nitrate concentrations whilst maintaining populations of all three species of amphibian found during the sampling programme.

3.3.3 Patterns of Nitrate Concentrations during Phase Two (1994-1995)

In the second phase of monitoring, the number of ponds sampled was increased to 53, compared to the six ponds and a ditch in 1994 (Appendix 3).

Initially, seven sites were randomly selected according to their ease of accessibility, out of the 53 sites, to monitor residual nitrate concentrations whilst maintaining a minimum work effort during the winter period. The first visit was on the 6 December 1994, with an additional visit to 9 sites randomly selected from 53 sites selected on the 20 January 1995. On the first visit, the majority of sites were 0 or 1 mg/l NO_3^- N, with residual nitrate levels on the second visit being greater than 20 mg/l NO_3^- N with one notable high level of 93 mg/l NO_3^- N recorded at one site: Gaulby Lane. It was decided that due to the high readings recorded during the second visit to the sites, that this would mark the start of the nitrate-monitoring programme for the 1995 season.

The majority of sites were fed directly by rainfall and surface runoff from the surrounding land (58%, n=30). Field drains were responsible for feeding water to a 25% of the sites. The remaining sites were fed either by springs, streams or ditches (Figure 3.7). Over the monitoring period (2 March to 22 June 1995), the average nitrate concentration recorded for sites fed by field land drains was 33.37 mg/l

NO_3^- N. For those sites fed by rainfall and runoff, the average was 2.78 mg/l NO_3^- N. The remaining sites fed by springs or streams, recorded an average nitrate concentration of 9.86 mg/l NO_3^- N (n=7). Frequencies of nitrate concentrations when spawns were first recorded are shown in Table 3.3 and presented in Figure 3.6, divided into groups according to the range of nitrate exposure.

Table 3.3 Nitrate concentrations (mg/l NO_3^- N) recorded on the 13 April 1995, across Phase Two pond sites (n=52).

	Nitrate concentration range (mg/l NO_3-N)						
Date	**0**	**1-10**	**11-20**	**21-30**	**31-40**	**41-50**	**51-200**
13 April 1995	35	3	0	3	2	1	8
Proportion (%)	.67	0.058	0	0.058	0.039	0.019	0.15

The measured concentrations of nitrate across all the sites on this day revealed that 67% of the sites recorded residual nitrate concentrations of 3 mg/l NO_3^- N or less. Ponds with nitrate concentrations more than 51 mg/l NO_3^- N accounted for 15% of sites, with the remaining 17.3% of ponds, having exposure concentrations between 1 and 50 mg/l NO_3^- N. By the end of April 1995, frog spawn was found at 50% of all sites sampled. Adult smooth newts were found in the ponds at 71% of sites.

Higher residual nitrate concentrations were associated with those sites fed by field drains. Lowest levels of nitrate were associated with those sites that whose water feed was from rainfall or runoff. Field drains recorded mean nitrate concentration on the 13 April 1995 of 33.37 mg/l NO_3^- N. This compared with the mean nitrate concentration recorded for rainfall and runoff fed sites of 2.78 mg/l NO_3^- N. From these observations, it is possible to conclude from this that higher nitrate concentrations in ponds were highly associated with the presence of field drains in the surrounding agricultural land. Where field drains were not present and the main feed source was by rainfall and runoff, lower residual nitrate concentrations were recorded. For the remaining sites, fed by either springs or streams, there appeared to be a similar distribution of sites across the range of nitrate bands.

Figure 3.6 Frequency of each nitrate concentration (mg/l NO_3-N) band on the 13 April 1995 (frequency = y axis; nitrate concentration range = x-axis).

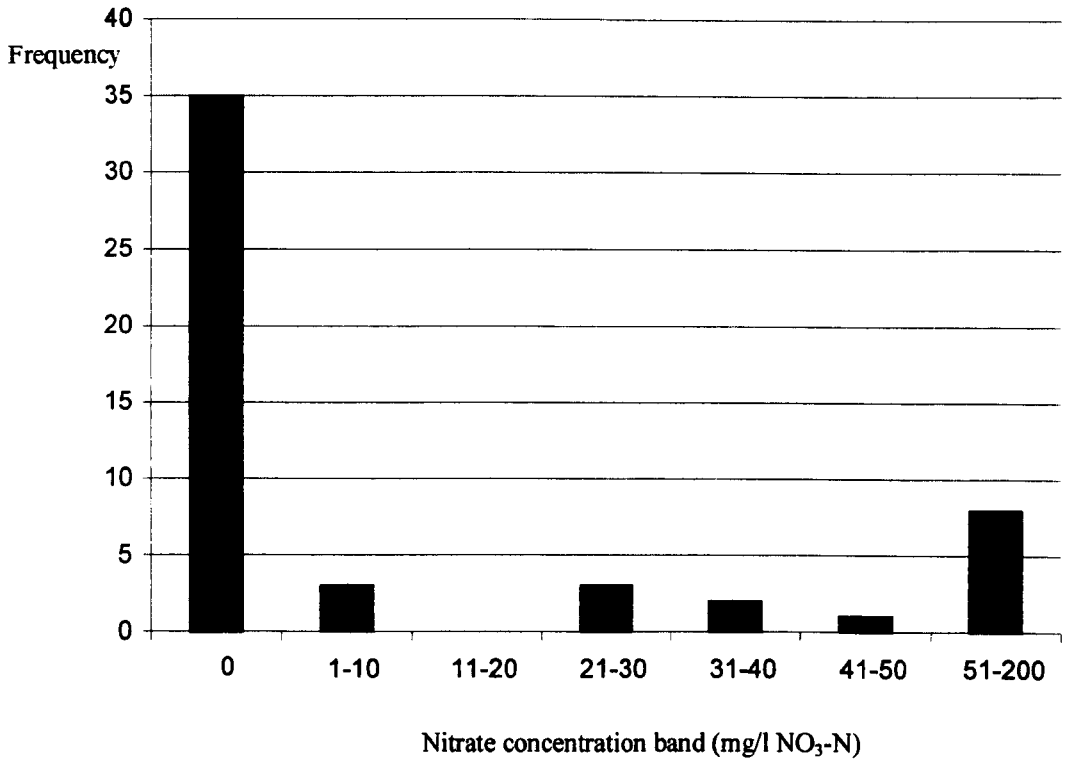
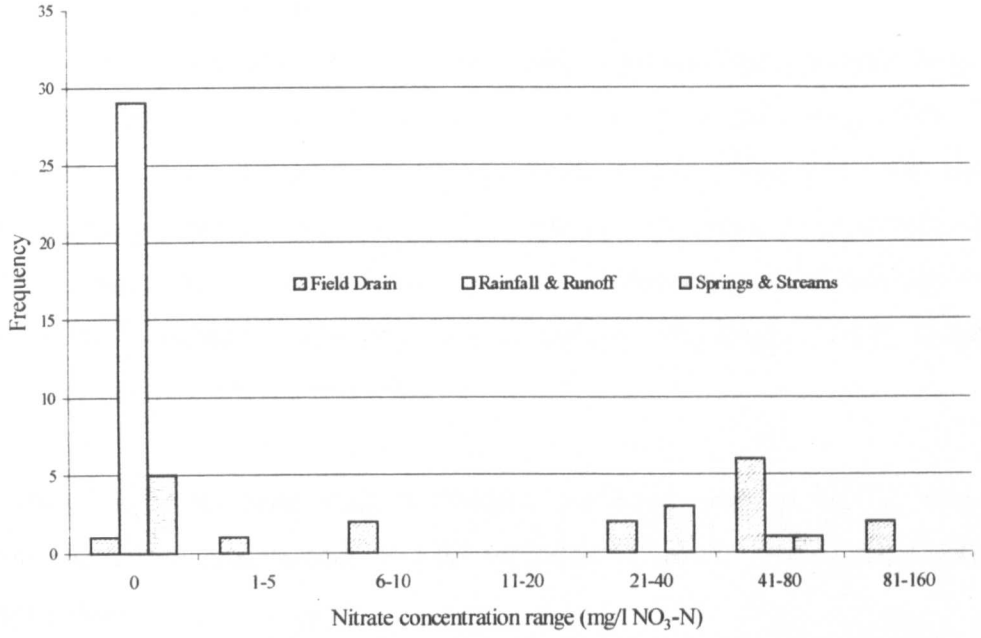


Figure 3.7 Frequency of each water supply source to all ponds divided acoording to their nitrate concentration band.



3.3.4 Common frog monitoring.

There was no correlation between the nitrate concentrations recorded in the field sites in January to April (1996) and the number of common frog spawn clumps counted at each site (n=24 sites) monitored during phase 2 of the sampling programme (January – April 1996). This time was chosen as it coincided with first observation of common frog spawns and was coincident with the early applications of nitrate fertiliser. This was tested using a Spearman' Rank Correlation coefficient (r_s) displayed in Table 3.4.

Table 3.4. Spearmans rank correlation coefficient dataset for the correlation between nitrate concentration and the incidence of spawn clumps during January – April 1996.

Variable x. Mean nitrate concentration (mg/L)	Rank of **x**	**Variable y.** Number of clumps counted	Rank of **y**	d	d^2
27.2	19	19	14	5	25
25.5	18	5	6	12	144
14.8	16	7	7.5	8.5	56.25
68	23	26	18.5	4.5	20.25
82.3	24	7	7.5	16.5	272.25
53.2	22	26	18.5	3.5	12.25
0	6	28	20	-14	196
0	6	85	25	-19	361
0	6	31	21	-15	225
0	6	2	3.5	2.5	6.25
0	6	32	22	-16	256
0	6	22	16	-10	100
49.5	21	2	3.5	17.5	306.25
0	6	1	1.5	4.5	20.25
0	6	23	17	-11	121
20	17	8	10	7	49
30.6	20	19	14	6	36
0	6	1	1.5	4.5	20.25
9.5	15	45	23	-8	64
0	6	4	5	1	1
0	6	10	12	-6	36
0	6	8	10	-4	16
1	14	50	24	-10	100
1	14	19	14	0	0
1	14	8	10	4	16
			$\varepsilon d^2 =$		2460

Therefore, $r_s = 1-[14760/13800]$

$= 1-1.06956$

$= -0.06$

The r_s value does not exceed the critical value of significance at the p=0.05 level (0.343). Therefore, we must accept that there is no significant correlation between the nitrate concentration and the occurrence of spawn clumps in ponds, and that clumps are as likely to occur in ponds with high residual nitrate compared with those with low residual nitrate concentrations.

The effect of the water supply source to each site and the proportions of each species of amphibian present at each site were analysed using sing chi – squared analysis. Table 3.5 shows a contingency table of frequency of each species recorded at each site type and the way in which the sites were supplied with water.

Table 3.5 Contingency Table of amphibian species in ponds relative to the water supply source.

Feed Source		Rana temporaria	Triturus cristatus	Triturus vulgaris	Total
Field Drain	Obs*	92	5	12	109
	Exp**	95.78	5.63	7.58	
	χ^2	(0.149)	(0.070)	(2.577)	
Rainfall & Runoff	Obs	304	18	18	340
	Exp	298.77	17.57	23.66	
	χ^2	(0.092)	(0.011)	(1.354)	
Springs, stream or ditches	Obs	46	3	5	54
	Exp	47.45	2.79	3.76	
	χ^2	(0.044)	(0.016)	(0.409)	
Total		442	26	35	503

*-Observed frequency; **-Expected frequency; χ^2-individual chi-square values

The majority of sites were fed by rainfall and runoff from surrounding land. These sites were associated with low residual nitrate concentrations. Field drains accounted for 22% of sites and 11% were fed by either spring or by stream. Common to all sites where amphibians were found, the common frog accounted for

the greatest proportion of amphibians found, ranging between 51 and 70% of the total number of amphibians found at each site.

Chi-square (χ^2)- analysis revealed that no significant associations existed between the water feed type and the species of amphibian recorded at each site ($\chi^2_4 = 4.72$, $P>0.05$). This appears to suggest that no site preferences were shown by the species found and the way in which water was supplied to each of the sites.

3.4 DISCUSSION.

Nitrate concentrations increased during the winter and spring, with concentrations of residual nitrate in the majority of sites decreasing during summer in both years of study. High concentrations of nitrate were recorded in ponds during the breeding season of not only the common frog, but also of smooth and crested newts (March and June). Common frogs were found as likely to occur in sites with high and low residual nitrate status. Sites fed by land drains recorded higher residual nitrate concentrations compared with those sites where water supply was through surface runoff, rainfall or stream water. There were no significant differences between the total numbers of a particular amphibian species found in relation to the residual nitrate concentrations, although a greater number of common frog spawns were found at sites supplied with rainfall and runoff supply water.

CHAPTER FOUR

THE IMPACT OF AMMONIUM NITRATE FERTILISER ON COMMON FROG (*Rana temporaria)* SPAWN

CHAPTER FOUR

THE IMPACT OF AMMONIUM NITRATE FERTILISER ON COMMON FROG (*Rana temporaria*) SPAWN.

4.1 INTRODUCTION

The ova of the common frog are laid in an aquatic environment encased within a protective jelly sac, as a spawn mass or clump. The morphology of the clump depends on adaptive differences between species according to their location, and the extremes of environmental conditions they may encounter (Moore, 1940). The jelly sac of *Rana temporaria* maintains the ovum temperature above that of the surrounding environment (Savage, 1951). Similar elevated temperatures have been observed in other species (Hassinger, 1970). In general, the common frog, during the breeding season (February to April in Britain), lays a single clump in the margins of ponds and ditches. Each clump contains between 800 and 2000 individual ova (Savage 1951). In the majority of anuran species, each individual jelly sac has up to five discrete membranes (Lee 1967). Common frogs are thought to have at least three outer membranes and one inner membrane (Deuchar, 1975; Salthe, 1963). The membrane directly surrounding the egg, known as the peri-vitelline membrane, and is produced by the ovary. The additional muccoidal membranes consist of long chain mucopolysaccahrides that surround the vitelline membrane and are secreted by the oviduct as the ovum passes through the reproductive tract. These membranes have been characterised through cytochemical staining (Freeman (1968). The complex chain structure of the membranes gives each a high internal pressure relative to the external environment of the surrounding water. This results in a diffusion gradient from the lower ionic concentrations of the external aquatic environment into the high ionic concentration within the jelly sac. Following oviposition, water enters by diffusion through microtubules within the discrete layers (Lee 1959), leading to the characteristic spawn mass (Salthe 1963). There is an increase of

approximately 15% in the sac volume before first cleavage of the developing zygote, approximately 2-3 hours after fertilisation. An additional 5% swelling occurs over the first 48 hours (Lee, 1959, Backmann, 1969).

In this chapter, the effect of spawn swelling of the jelly sac membranes were investigated by exposing sub samples (sub-clumps) of common frog spawn to solutions at different concentrations of ammonium nitrate fertiliser under laboratory conditions. To achieve this, spawn collected from the field was divided and exposed to fertiliser in solution at different concentrations. The amount of swelling of jelly sacs and the impact on the developing embryos was established by measuring hatching success and physical parameters of zygote and jelly sac diameters.

4.2 METHODS.

4.2.1 Investigation One: Acute (24 hour) exposure of spawn to moderate concentrations of ammonium nitrate (50 mg/L NO_3^-N)

The first investigation was used to establish if common frogspawn would be at risk from exposure to a single concentration of ammonium nitrate in solution. This would simulate the effect of a pulse episode of nitrate pollution in the field. A clump of spawn was collected from a local site, Woodpond (SK 665053). This site was selected for the collection of frogspawn because the surrounding land had a history of low intensity farming (Renner N, pers comm.)

The pond was visited each morning from the start of January 1994 until mid February 1994. Residual nitrate (determined using a handheld BDH-MERCK RQ-Flex colorimeter) in daily samples of water taken from the pond revealed that concentrations of nitrate were lower than 3 mg/L NO_3^-N, for the entire six week period before the investigation. In the third week of February, a single clump of

spawn was collected from Woodpond and returned to the laboratory. The water temperature of the pond on collection was approximately 6°C. The temperature of the fridge was held at a similar temperature to that of the pond at time of collection, to avoid any possible damage to the developing ova from temperature shock. The spawn was placed into a plastic 10 litre clear aquarium, with approximately 6 litres of aged tap water (at a depth *ca.*10cm) for an hour, held in a fridge at 6°C. To obtain aged tap water, chlorinated tap water was transferred from a steel water tank into Nalgene plastic aspirators (50 litres) for at least 24 hours before use. The water was aerated and kept in a darkened room to reduce alga and bacterial growth. Room temperature was kept at approximately 20°C. Residual nitrate concentration of aerated aged tap water was determined using the handheld colorimeter as between 5 and 9 mg/L NO_3^-N.

When suspended in water, the number of eggs held in the clump was estimated by eye to be between 1500 and 2000. Following the first hour of acclimation in the fridge, the clump was transferred gently by hand, from the aquarium and placed into a shallow tray containing cooled aged tap water. Cell viability was assessed by observation under binocular stereoscope for signs of development. Clear cell division with vegetal and animal hemispheres indicated that development was approximately at stage 8-10 (Gosner, 1960) and 10 hours old. Ova with a marbled, speckled appearance, or obvious rupturing were removed using a large bore glass pipette, gently drawing the ruptured or necrotic ova into the pipette and discarding. The main clump was divided carefully by hand into four equal parts manipulated by using blunt forceps and gloved hands. Two quarters (A & B) were placed into a 10-litre plastic aquarium containing 5 litres of aged tap water and returned to the fridge. The third quarter (C) was placed into a plastic aquarium containing 5 litres of aged tap water, and acted as the control clump. The volume of water in the aquariua was sufficient to allow the clumps to float clear of the bottom of the tank. The final quarter (D) was placed into 5 litres of fertiliser solution prepared at a nominal 50 mg/L NO_3^-N (with aged tap water). The approximate egg and jelly sac diameters were recorded for both clumps

before and after 24 hours, by measuring 10 randomly selected peripheral ova from each clump. The diameter of the zygote and the diameter of individual sacs were established by measuring the distance between the centers of two adjacent developing zygotes. This was achieved by suspending the main clump, to reduce the amount of distortion of the egg sacs that would be associated with measurements on a flat surface. Measurements were achieved using a pair of vernier calipers placed as close as possible to the developing zygote without damaging the jelly sac. Clump quarters C & D were held under conditions of constant light and temperature (6°C) in a fridge for 24 hours allowing peripheral egg sacs to float in the cooled aged tap water in the tray. This method was limited by the number of clumps that were available for use at such a late stage in the season. It is therefore accepted that although poor replication weakened the investigation statistically, it nevertheless demonstrated the impact of ammonium nitrate on a large number of individually developing zygotes from similar clumps. Sampling spawns from different geographical areas could have assessed spacial differences. This would also have demonstrated whether populations selected with different genetic backgrounds were similarly affected.

4.2.2 Investigation Two: Chronic (17-day) exposure of spawn to a range of ammonium nitrate concentrations in solution

An additional investigation was undertaken to establish the impact of ammonium nitrate in solution on the embryos of the common frog over a prolonged exposure period. This was carried out using the remaining two quarters (A & B) of the divided main clump collected and isolated during Investigation one. The two quarters were divided into 20 smaller sub-clumps by hand, (each of approximately 10g), and exposed to 5, 10 20, 40 and 80 mg/L nominal ammonium nitrate concentrations. The sub-clumps were checked for viability, removing non-viable eggs in the same manner as before. Following division, the smaller clumps were transferred into a single 10 litre plastic aquarium containing 7 litres of aerated-

aged tap water at approximately 6°C. Twelve individual clumps were then randomly selected from the aquarium. Their masses (g) were determined using a top pan balance. The number of ova in each clump was counted. The jelly sac and ovum diameters were measured as before. Sub-clumps were then randomly assigned to replicate exposure vessels (1 litre glass beakers containing 500 ml of test media) at 5, 10, 20, 40 and 80 mg/L nominal concentrations as $NH^{+}_{4}NO^{-}_{3}$, each vessel prepared in duplicate. Two controls were prepared with chilled (ca. 6°C) aged tap water, giving a total of 12 vessels. All beakers were placed in a fridge at 6°C. The spawn clumps were held in the fridge under conditions of constant light and temperature for 5 days, during which, there was a daily renewal of ammonium nitrate solution at each concentration. During the 5 days incubation, the fridge temperature was increased daily by 2°C until control water temperature was between 11-16°C. Room temperature was avoided as research has shown that at *ca.* 20°C, although developmental rates are accelerated, hatching success is greatly reduced (16%), where as at between 11 and 16°C, hatching success was almost 100% (Grainger 1958). Solutions of fertiliser were renewed every 24-hours until the first hatch was observed in the control embryos. At this point, the numbers of surviving larvae in all containers were counted. The mass and diameter of each clump was determined as before. The spawn clumps were then left at ca. 16°C under conditions of constant light, to promote the hatching of the remaining embryos from all treatments. In addition to ova and jelly sac diameter measurements, percentage survival values at each exposure concentration and relative sub clump masses were recorded for each exposure concentration. Ova and jelly sac diameters were measured from 3 randomly selected embryos on days 0 and 17 at each exposure concentration. All clumps were left for an additional four days at 16°C to allow surviving embryos to hatch to assess survival. As for investigation one, it is accepted that there was poor replication in the test design, again for similar reasons as already stated. By keeping to single clumps and in effect pseudo-replicating within the test design, the effects statistically would be weakened when compared to field situations. However they do demonstrate the potential risk and attempt to quantify the affect

of ammonium nitrate on developing embryos across a range of nitrate concentrations.

4.3 RESULTS

4.3.1 Investigation One: Acute (24 hour) exposure of spawn to moderate concentrations of ammonium nitrate (50 mg/L NO_3^-N)

Tables 4.1 and 4.2 show the measurements of jelly sac and embryo before and after the 24 hour exposure period. After 24 hours, the jelly sacs exposed to 50 mg/L ammonium nitrate had increased significantly between the control and the exposed clumps of spawn ((t test) $p=<0.01$, d.f. = 9), with no increase in the embryo diameter ((t test)$p=0.79$, d.f.= 9) over the same period. The jelly sac diameter of the exposed half of the clump visibly increased in diameter.

Table 4.1. Measurements of developing ova and surrounding jelly sac diameters (mm) before exposure to ammonium nitrate according to assigned treatment groups.

Treatment group	Ova-diameter (mm)	Jelly sac diameter (mm)
Control	5.2 ± 0.52*	15 ± 0.71
50 mg/L NO_3^-N	5.1 ± 0.42	14.9 ± 0.56

* Mean values shown with standard errors

Table 4.2. Measurements of developing ova and surrounding jelly sac diameters (mm) following 24 hours exposure to 50 mg/L NO_3^-N.

Treatment Group	Ova diameter (mm)	Jelly sac diameter (mm)
Control	5.6 ± 0.52*	15.8 ± 0.71
50 mg/L NO_3^-N	5.1 ± 0.42	25.5 ± 0.56

* Mean values shown with standard errors

Jelly sac diameters increased in both the control and 50 mg/L NO_3^-N exposed clumps. An increase of approximately 0.6 mm was recorded for the control jelly

sacs, with an increase of 10.5 mm being recorded for the clumps exposed at 50 mg/L NO_3^- N. The diameter of the ova, over such a short exposure period remained unchanged.

4.3.2 Investigation Two: Chronic (17 day) exposure of spawn clumps to a range of ammonium nitrate concentration (5, 10, 20, 40 & 80 mg/L NO_3^- N)

Hatching in the control larvae was observed at 400 hours after the collection of the spawn from the control sites. At 400 hours, clumps exposed at higher concentrations were visibly more swollen in comparison to the control clumps. Table 4.3. shows the result of the egg swelling exposure test on day and on day 17.

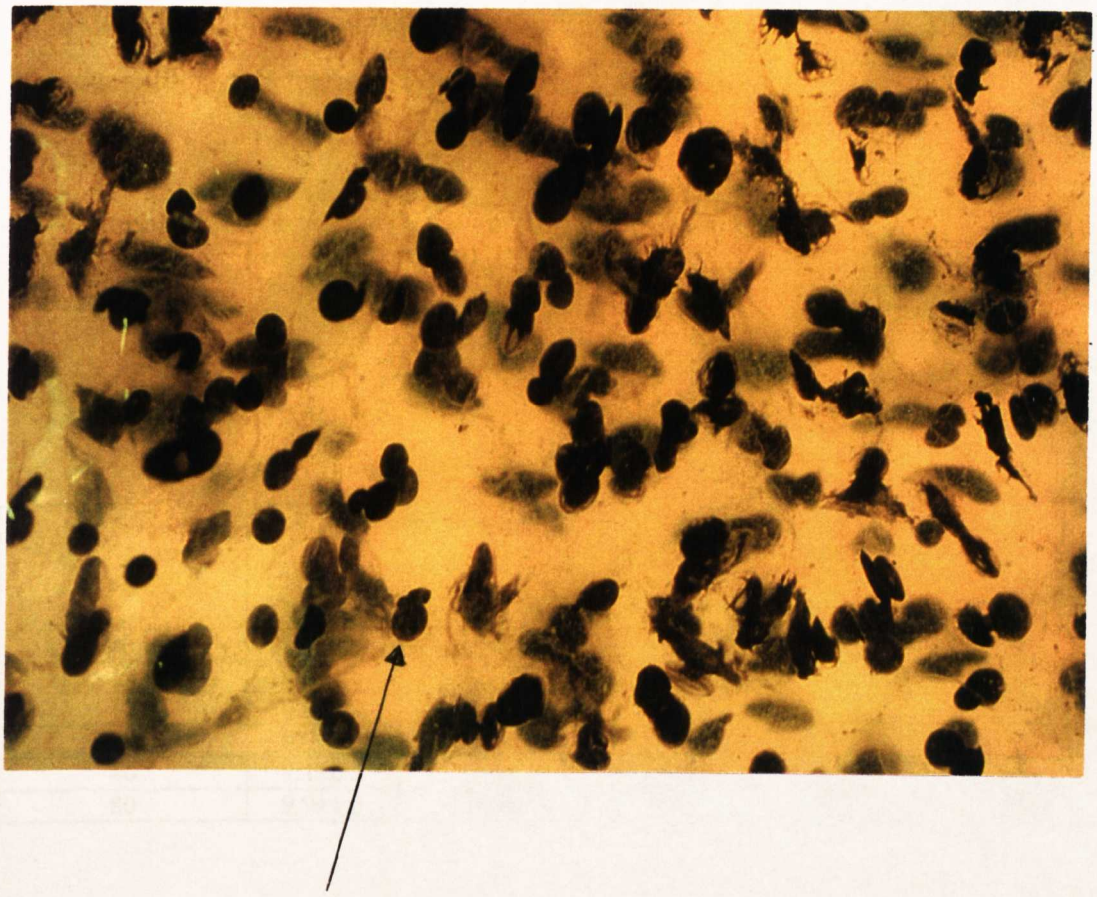
Table 4.3. Day 0 and Day 17 mean ova and jelly sac measurements for spawn exposed to ammonium nitrate at 5, 10, 20, 40 and 80 mg/L NO_3^-N.

Nominal $NH^+_4NO^-_3$ Conc.,mg/l	Mean measurements (mm)			
	Day 0		Day 17	
	Ova diameter	Jelly sac diameter	Ova diameter	Jelly sac diameter
Control	4.8 ± 0.33	14.2 ± 0.32	6.0 ± 0.09	16.2 ± 0.25
5	5.2 ± 0.07	14.9 ± 0.19	6.5 ± 0.06	18.5 ± 0.40
10	5.3 ± 0.14	15.0 ± 0.14	6.5 ± 0.04	18.2 ± 0.35
20	5.0 ± 0.07	12.3 ± 0.13	5.5 ± 0.22	22.9 ± 0.64
40	5.3 ± 0.09	14.7 ± 0.76	6.4 ± 0.12	26.2 ± 1.07
80	5.4 ± 0.07	14.3 ± 0.63	5.6 ± 0.13	21.1 ± 0.60

Standard error shown, N=6

Table 4.3 shows that over the 17-day duration of the experiment, ammonium nitrate fertiliser in solution had an effect on the developing ova and the jelly sac measurements. Analysis of variance revealed that for developing ova, nitrate concentration was found to have a significant effect on ovum diameter (ANOVA; $F=6.48$, $p<0.01$). There was no difference between the jelly sac diameter between replicates ($p=0.964$). However there was significance differences between the

Plate 4.1 Common frog spawns exposed at 40 mg/L NO^{-}_{3}-N at developmental stage 17-18, showing ruptured spawn sacs.



jelly sac diameters. A Tukey test on the differences between the means across the data set in conjunction with the within group sum of variances, gave a T statistic of T = 5.7. From this value it was established that jelly sacs exposed at 40 mg/L ammonium nitrate were significantly different at the p =0.05. From the data, this is shown as a significant increase in diameter relative to the control treatment.

The impact of ammonium nitrate treatment on hatching success was assessed. Tables 4.4 and 4.5 show that on each count day, the number of viable eggs in all ammonium nitrate treatments were shown to decrease.

On day 21, after all viable larvae had hatched, there was 87% hatching success in the control clumps. This compared with 63% observed at the highest NO_3^- N concentration.

Table 4.4. Investigation 2. Mean (+ s.e.) sub-clump masses (g) and viable ova counts for sub clumps exposed to ammonium nitrate.

Nominal $NH^+_4NO^-_3$ conc. (mg/l)	**Sub-clump mass(g)**		**Sub-clump viable ova count (Larval count on day 21).**		
	Day 0	**Day 17**	**Day 0**	**Day 17**	**Day 21**
Control	8.3±0.7	69.4±5.4	46±5	44±6	40±6
5	9.8±1.6	60.7±1.7	52.5±9.5	49±10	47.5±9.5
10	9.5±2.1	77.9±7.3	55±9	49±12	48.5±11.5
20	9.9±0.3	73.9±2.5	65±7	58.5±5.5	54±7
40	9.1±0.3	77.3±1.2	40.5±1.5	33±1	31±1
80	9.9±1.0	30.4±6.9	43±8	32±5	27±4

Table 4.5. Investigation 2. Number of viable eggs shown as a percentage of the Day 0 viable egg count and the developmental stage according to Gosner (1960) at each ammonium nitrate exposure concentration.

Nominal $NH^+_4NO^-_3$ conc.(mg/l)	Viable embryos*		Developmental stage (Gosner 1960)	
	Day 17	Day 21	Day 17	Day 21
Control	95	87	19	20
5	93	90	18	20
10	89	88	18	19
20	90	83	18	19
40	81	76	18	19
80	74	63	17	18

*The overall number of viable embryos from the two replicates at each ammonium nitrate treatment expressed as percentage of the overall starting (Day 0) number of viable embryos.

Inspection of the spawn clumps at 40 mg/L and 80 mg/L NO_3-N revealed in both cases egg capsules that had ruptured releasing the contents of the sacs into the external media (Plate 4.1).

4.4 DISCUSSION

Ammonium nitrate in solution has been shown to have a significant affect on jelly sac diameter, ova diameter, embryo viability and subsequent hatching success. Exposure to 50 mg/L ammonium nitrate in solution for 24 hours had the effect of increasing jelly sac diameter significantly. Mean pre-exposure jelly sac diameters were 14.9 ± 0.6 mm. Compared with the mean post-exposure jelly sac diameter of 25.5 ± 0.8 mm ($p<0.01$, d.f. 9). There was no impact on the diameter of the developing ova. Low level exposure to ammonium nitrate, up to 10mg/l nominal nitrate concentration in solution had little or no impact on jelly sac or ovum diameter. However, increasing concentration of ammonium nitrate saw an increase in jelly sac diameter. After 17 days exposure ovum viability was reduced at the higher nitrate concentrations of 20, 40 and 80 mg/L NO^-_3 N. After 21 days, hatching success was significantly reduced at 40 and 80 mg/L NO^-_3 N, relative to the control clumps. Clump mass (g) was seen to increase with increasing ammonium nitrate concentration relative to the control clump masses. This was due to an increase in the volume of water held in the jelly sacs. Over the period of exposure, all clump masses were observed to increase in size, with the exception of the 80 mg/L NO^-_3 N treatment that did not appear to expand relative to the lower levels of NO^-_3 in the test. In exposed clumps, ovum diameters appeared to reduce significantly throughout the test, with the majority of elongation in the exposed clumps occurring after day 17 of the test. The main impacts were observed in the swelling of the jelly sac with the jelly sac diameter being a measure of inter-egg distances. These were seen to increase with increasing ammonium nitrate concentration relative to the control clumps. The number of surviving embryos at day 17 was reduced as ammonium nitrate concentration increased. On day 21, the total number of hatched larvae that survived was seen to decrease with increasing nitrate concentration. This implies that as the concentration of ammonium nitrate increases, the osmotic nature of the external media relative to the internal environment of the jelly sac leads to a net-flux or diffusion of external media into the jelly sac (Lee, 1967). As an increased amount of water diffuses into the jelly sac, the integrity of the membranes begins

to break down, leading to the membrane rupturing. Of the embryos that survived at higher nitrate concentrations, there was reduced hatching success, which was positively correlated with increased jelly sac diameter.

Research on the impact of pH on the hatching success of ranids and bufonids has suggested that to hatch successfully, the volume of the peri-vitelline fluid surrounding the developing embryo must increase. Over the developmental period of the embryo, the increase in volume leads to a break down of the membranous layers within the jelly sac. Larval movement and the release of hatching enzymes facilitate hatching.. In *Xenopus laevis*, at pH = 4.0 the hatching enzyme is completely inhibited, preventing hatching (Leuven et al, 1986). Clump mass was lower at highest concentration of nitrate (80 mg/L NO^-_3 N). The jelly sac integrity at this concentration was breached, with embryos present at the bottom of the vessel. Where ruptured jelly sacs were present, embryo survival was drastically reduced.

From these findings, ponds exposed to sources of high nitrate concentrations through ground water, greater than 40 mg/L NO^-_3 N, there is the potential for reduced hatching success from spawn clumps laid at these sites. Spawn deposited in ponds and ditches with high nitrate status are likely to have a poor jelly sac integrity and therefore poor survival. What these investigations do not consider are the role of other ions in the field and their effects on jelly sac integrity and subsequent zygote development. Additional treatments using calcium and sodium nitrate would have been useful to include into the test design. Their use could further elucidate the role of increasing ion concentration on the expanding properties of jelly sacs, giving a clearer picture of risk in the field.

Investigations using Salmonid eggs and fish fry have revealed high mortality rates following a 30 day exposure to NO^-_3 at concentrations up to 30 mg/L NO^-_3 N (approximately 6.6 mg/L NO_3^-N) using sodium nitrate as the nitrate source. High levels of egg mortality (15-20%) occurred at 10 mg/L NO^-_3 N. Fry mortality was

seen to be slightly less, with between 10 and 15% mortality for a number of salmonid species including Rainbow Trout and Coho Salmon. (Kincheloe *et al*, 1979). These levels of mortality were reported knowing that nitrate was considered non-toxic to salmonid fingerlings (EPA, 1976) with 96 hr TLm (threshold limit of mortality) of 6000 mg/L for rainbow trout fingerlings. This reinforces the requirement for studies using earlier life stages, such as the fish egg and juvenile fish fry investigations, which may be more sensitive to the effects of otherwise non-toxic chemicals.

CHAPTER FIVE

THE TOXICITY OF AMMONIUM NITRATE FERTILISER TO LARVAE OF THE COMMON FROG (*Rana temporaria)*

CHAPTER FIVE

THE TOXICITY OF AMMONIUM NITRATE FERTILISER TO LARVAE OF THE COMMON FROG *Rana temporaria*

5.1 INTRODUCTION

The aim of this Chapter is to establish the effective and lethal concentrations (EC_{50} and LC_{50}) of ammonium nitrate in solution on developing common frog larvae. A dose response relationship will be established for ammonium nitrate and the common frog, to provide the basis for hazard assessment. The toxic effect of a range of concentrations will be tested under laboratory conditions. Many toxicological methods establish the toxicity of chemicals by establishing end-points (usually death) in a proportion of a test species. The EC_{50} is the corresponding concentration of a test material that will cause an effect in 50% of individuals in a test population. An effect may manifest as a change in behaviour, such as a loss of coordination whilst swimming, lethargy, lack of response to stimulation. Alternatively, physical changes may occur, such as pigmentation or colour change. The LC_{50} is the corresponding concentration that causes death in at least 50% of individuals within a test population. By establishing these values, it is possible to establish the highest concentration that causes no effect (no observed effect concentration – NOEC) and the concentration which causes 100% effect or death in a test population.

These values will be used as the basis for setting test concentrations in the longer-term investigations in Chapter Six.

The life history of the common frog is dependent on the aquatic environment. This dependence places populations of common frogs at risk from a range of chemical pollutants. Herbicides, detergents, insecticides, and fertilisers all have the potential to pollute and persist within aquatic environments and with the result of modern agricultural practices.

Aquatic habitats associated with breeding common frog populations exist in rural environments. The breeding season occurs during a season when water levels in ponds is high, and at a time when the application of chemicals to land adjacent to these ponds is occurring. The water that runs off from fields carries a high burden of pollutants via land drains and surface runoff into the water bodies. This places frog populations under pressure by having polluting chemicals present in the water body at a time when individuals are most susceptible, namely the period of early growth and development as larvae.

When studying physiological and anatomical impacts, the advantage of using frogspawn is apparent with large ova and individuals all of similar stages of development. These make the study of physiological parameters relatively simple, with many developmental stages easily distinguishable by eye.

The effect of chemical contamination includes the effects of metals in the aquatic system. Lead pollution in the aquatic phase has been shown to affect the acquisition of learning in *Rana clamitans* tadpoles to changes in an individual's ability to show positive preference and avoidance behaviour (Taylor, Steel, Strickler and Shaw, 1990). Low levels of pH in natal breeding sites water produced 100% mortality in common frog larvae (Ling et al, 1986). Beattie *et al.* (1992) observed spatial and temporal differences in levels of survival between upland, and lowland populations of common frog larvae in pond waters of similar pH level. They demonstrated that individual larvae have the ability to adapt to small changes in pH. The effects of low pH on common frog larvae within isolated natural upland and lowland ponds suggested that tolerances to differing environmental conditions within the field do exist. Physiologically, low pH leads to a thickening of vitelline membranes of the spawn and the inhibition of essential hatching enzymes (Freda, 1986) leading to low hatching success and poor survival. In newly hatched larvae, low pH caused a loss of sodium from the body of common frog larvae, with losses greater than 50% being lethal (Warner *et al.* 1991). However, consideration of the effects of fertilisers on other organisms

may be helpful. For example, studies into mortality in ruminants revealed that a high level of nitrate in ruminant feed led to high levels of nitrite in the circulation. This had the effect of reducing gaseous exchange across essential membranes, leading to abnormal rates of mortality. Subsequent investigations revealed that in those animals that had died, there were significant increases in the size of the thyroid glands. This suggests that nitrate may indirectly interfere with the essential uptake of iodine by the thyroid gland, leading to hypothyroidism (Emerick, 1974).

The effect of ammonium nitrate fertiliser on common frog larvae has not been extensively studied. Some research has revealed that sodium nitrate has been shown to reduce larval growth rates in the common toad, *Bufo bufo* at 40 and 100 mg/L NO^-_3 N, with higher levels of mortality at the higher concentration. (Baker, 1993). Nitrate was also shown to decrease growth rates of *Littoria caerulea* tadpoles and increase levels of mortality when exposed to sodium nitrate at 40 and 100 mg/L NO^-_3 N relative to sodium chloride controls and dilution water controls (Baker & Waights 1994).

Ammonium nitrate has been shown to reduce the activity levels of common toad larvae at 100mg/l NO^-_3 N (Xu & Oldham, 1996), but no clear effects on growth rates, food consumption, or developmental rates were observed. LC_{50} values of 1704 and 1637 mg/L NO^-_3 N were recorded as 96 and 168-hour LC_{50} values. Toad larvae in all treatments groups were observed with anatomical deformities and changes in swimming behaviour.

Smooth newt larvae exposed to ammonium nitrate at 200 and 500 mg/L as ammonium nitrate were significantly smaller than control larvae at metamorphosis. Larvae exposed to ammonium nitrate at 100 mg/L NO^-_3 N had significantly greater feeding rates than controls (Watt & Oldham 1995).

Hecnar (1995) suggested that nitrogen based fertilisers may be a contributing factor in the decline of amphibian populations in agricultural lands in Canada (Hecnar, 1995).

Species differences in the sensitivity of larvae to nitrate and nitrite ions using chemical based fertilisers were detected in five species of amphibian, namely *Rana pretiosa, Rana aurora, Bufo boreas, Hyla regilla* and *Ambystoma gracile.* In some cases, species showed reduced feeding activity, loss of co-ordination increased incidence of abnormalities and increased rate of mortality with concentration (up to 50 mg/L $NO^{-}_{3}N$ and time (Marco *et al.* 1999).

This chapter aims to characterise the risk of ammonium nitrate pollution to common frog tadpoles in the aquatic environment were exposed under controlled conditions in the laboratory to sub-lethal levels of fertiliser. The impact on embryological and larval development and change associated with metamorphosis and behaviour, were assessed.

5.2 METHODS

Developing frog embryos and larvae were exposed to sub-lethal levels of ammonium nitrate in solution for acute (96 hours) investigations. Constant conditions of light and temperature were maintained. A series of preliminary toxicity tests were carried out to determine the effective and lethal concentrations of the fertiliser in solution using early staged common frog larvae. These tests were carried out under static test conditions. The results of these tests were used in conjunction with data on the residual concentrations of nitrate in the field to establish the test concentrations for the flow-through investigations.

5.2.1 Preliminary toxicity trials to establish an LC_{50} value under static test conditions in solutions of ammonium nitrate prepared in artificial pond water.

Preliminary tests were undertaken to establish the range of exposure concentrations to be used in the long-term flow-through laboratory investigations. The effective (EC_{50}) and lethal (LC_{50}) concentrations of ammonium nitrate in solution were established in static experiments (no test media renewal), where common frog larvae were exposed to ammonium nitrate solutions for 96 hour periods. Values for EC_{50} and LC_{50} were calculated using probit analysis. Probit analysis uses a regression analysis of the exposure concentration against response. The data are checked for normality using a chi-squared analysis, and then (if required) the exposure concentrations are transformed to normalise the data. The 50% response percentile is entered into the regression equation, giving a value for the EC_{50} or the LC_{50} respectively. These values are de-transformed from logarithms to give the concentrations as required.

5.2.2 Collection and incubation of common frog spawn

Four spawn clumps (developmental stage 6 (Gosner, 1960)) were collected from a control pond (residual nitrate concentration <3 mg/L NO_3) at the end of February 1995. The site (Woodpond (grid reference: SK 665053)) was monitored for the presence of common frogs and frog egg masses from the beginning of February 1995. This site was chosen due to its close proximity to the laboratories and a history of low intensity farming practice during the previous 3-5 years (Nelson Renner pers comm. (1995). Concentrations of nitrate (NO^-_3) were determined using colorimetry. Water temperature at the pond site was measured using a digital thermometer, with a sample of water being returned for pH determination in the laboratory. Spawn clumps were transferred to the laboratory in approximately 5-litres of pond water in 8-litre plastic buckets. Spawn was equilibrated to laboratory conditions over 24 hours by placing them into a fridge (9 ± 2°C) under constant light (<100 Lux). After the equilibration period, the four clumps were transferred to individual 10-litre plastic aquariums using the pond

water in which they were transferred to the laboratory. This volume was supplemented with artificial pond water (Appendix 1) to a final volume of 6 litres in each aquarium. Artificial pond water was held in the refrigerator for the same time as the spawns and the pond water. The clumps were then transferred to an air-conditioned facility, where environmental conditions were 16 hours dark and 8 hours light at 14-16°C for 6 days after which time the first viable embryos started to hatch. All embryos from all four clumps were hatched and free swimming (approximate stage 22-23 (Gosner, 1960)) after 15 days incubation. The hatched larvae were counted. All larvae were then pooled into two 30-litre glass aquaria containing approximately 25 litres of artificial pond water. Each aquarium was aerated using an aquarium pump. Water quality parameters were monitored (maintaining pH > 7.8 and < 8.5, dissolved oxygen (%air saturated value) was maintained at levels >80% with total hardness maintained at moderately hard levels *ca.* 200mg/l $CaCO_3$).

5.2.3 Larval Feeding Rates.

Larvae were fed using Tetramin® Floating Fish Food sticks at an arbitrary daily feeding rate of 2% of the mean wet weight of 10 larvae from each of the aquarium. This rate was based on the recommendations made for feeding of larval stock fish in the 1987 OECD Guideline Number 203: Fish, Acute Toxicity to Fish. In addition, a food supplement of boiled lettuce leaves was added to each aquarium *ad libitum*. This was utilised both as an additional food source and as refuge for the developing larvae. Feeding rates of dry food were established using Equation 1, showing the feeding rates per day for the two aquariums.

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The test duration for the EC_{50} determinations was 48 hours and 96 hours for the lethality tests (LC_{50}).

The effect criteria for the EC_{50} tests were a reduced level of activity relative to control larvae. This was indicated by individuals becoming lethargic or not responding to a blunt point stimulus, with individuals remaining motionless for extended periods of time (>30 seconds). Numbers affected were recorded at each concentration. Loss of co-ordination was also used to monitor effects, and manifested by individual larvae spiral swimming compared to control larvae whose swimming activity was in straight lines.

A lethal effect was indicated by lack of movement following a blunt point stimulus to the tip of the tail and confirmed by cessation of blood circulation in the caudal veins visible at the junction of the body and tail using a hand held (x10) lens.

Typical water quality for the batches of artificial pond water were pH = 7.9, dissolved oxygen was always greater than 8.9mg/l O_2, but not > 11mg/l, with water hardness between 192 and 205 mg/L $CaCO_3$ and temperature maintained 16 °C ±2 °C. Larvae (n=500) were selected from the stock aquariums and transferred to a pre-exposure holding tank. Developmental stage 23 (Gosner, 1960) was confirmed by observing individuals under a microscope before being used in the tests. In the preliminary test, 60 larvae were exposed. In the definitive test, 100 larvae were exposed. The testing regime was static and the test duration was 48 hours for an affect and 96 hours for lethality data. Results were expressed as percentage effect or kill for each exposure concentration. Values for EC_{50} and LC_{50} were established using a probit analysis.

5.3 RESULTS

5.3.1 Preliminary EC_{50} and LC_{50} toxicity tests

Table 5.2 shows the results of the initial toxicity tests showing the percent of larvae killed or exhibiting an effect for each time point.

Table 5.2 Results of the preliminary EC_{50} and LC_{50} toxicity tests. (n=60)

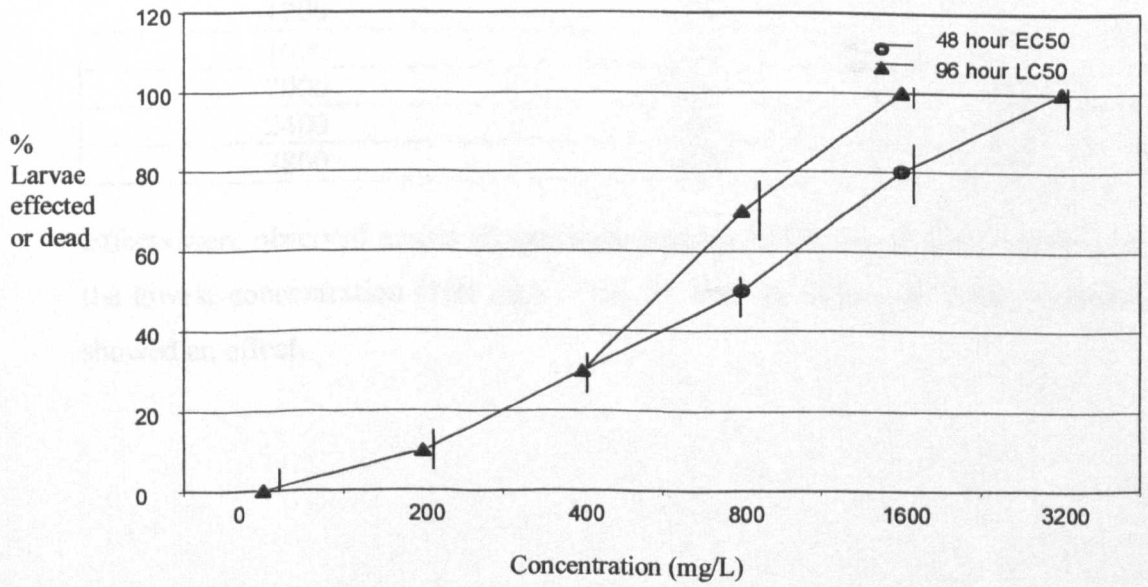
Nominal conc. ammonium nitrate (mg/l) as nitrate	Percentage of larvae affected (EC_{50}) after 48 hours.	Percentage mortality (LC_{50}) after 96 hrs
0	0	0
200	10	10
400	30	30
800	50	70
1600	80	100
3200	100	100

The results show that there was a dose dependent effect of ammonium nitrate on the larvae.

EC_{50}= 517.7 mg/L NO^-_3 N (95% confidence intervals of 441.6 & 1027.7mg/l).

LC_{50}= 683 mg/L NO^-_3 N (95% confidence intervals of 361 & 725.8mg/l).

Figure 5. .1 Dose response curve for larvae exposed during the preliminary test to concentrations at 0, 200 – 3200 mg/L NO^-_3 N.



5.3.2 Definitive toxicity test for common frog larvae

A definitive toxicity test was undertaken using concentrations that included the range of concentrations established in the preliminary toxicity test (0 to 3200 mg/L NO^-_3 N). This was carried out to ensure that a 100% effect could be demonstrated and that a NOEC 'no observed effect concentration' could be achieved. This is the maximum concentration at which no effect on the developing larvae could be detected.

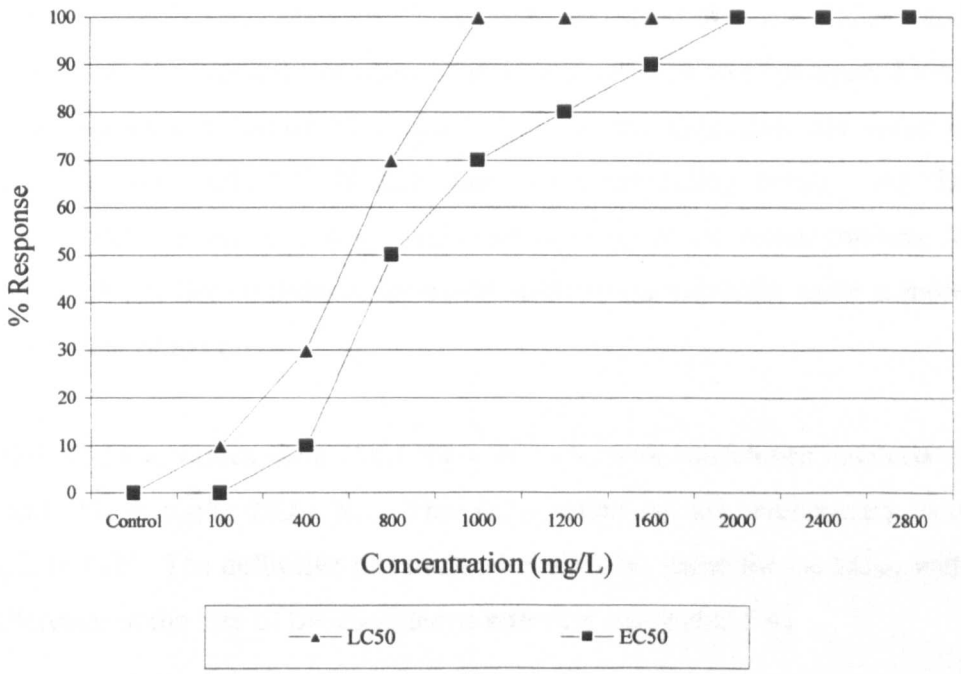
The number of exposure concentrations was increased in the definitive experiment in an attempt to reduce the size of the confidence intervals, whilst maintaining the same number of individuals exposed at each exposure concentration. The concentrations selected and the results of the definitive test are shown in Table 5.3 and Figure 5.1.1

Table 5.3 Definitive test results showing cumulative effects and percentage mortality (n= 100).

Nominal concentration of ammonium nitrate (mg/l as nitrate)	% of effected larvae after 48 hrs.	% mortality after 96 hrs.
Control	0	0
100	10	0
400	30	10
800	70	50
1000	100	70
1200	100	80
1600	100	90
2000	100	100
2400	100	100
2800	100	100

Effects were observed across all exposure concentrations except the controls. At the lowest concentration (100 mg/L NO^-_3 N) one out of the ten larvae exposed showed an effect.

Figure 5.1.1 Dose-response curves for EC_{50} (O) and LC_{50} (X) results for common frog larvae exposed to a range of ammonium nitrate concentrations (x-axis) with percentage of individuals affected (EC_{50}) or dead (LC_{50}) on the y-axis.



5.4 DISCUSSION

No observed effects were detected at 100 mg/L NO3-N. This concentration is based on observation of the data was considered to be the NOEC concentration. The 48 hour EC_{50} (determined using the probit analysis (Stephen *et al.*) was 398.67 mg/L NO^-_3 N, with 95% confidence intervals of 233.6 and 545.8 mg/L NO^-_3 N. When the results of the definitive study were compared with the preliminary EC_{50} value of 517.7 mg/L NO^-_3 N, the definitive test value is approximately 100 mg/L NO^-_3N less than the rangefinding result. As the definitive toxicity tested across a much narrower range of concentrations, it produced much smaller confidence intervals, making the resulting value a more realistic estimate of toxicity.

The definitive LC_{50} values were 781.1 mg/L NO^-_3 N, with confidence intervals of 586.9 and 942.4 mg/L NO^-_3 N. The LC_{50} value in the preliminary was 688 mg/L NO^-_3 N. The definitive test resulted in a higher value for the LC_{50}, with little difference in the size of the confidence intervals (see Table 5.4).

Table 5.4. Summary of toxicity test results for EC_{50} and LC_{50} determinations using common frog larvae.

Test	Toxicity test results (mg/l NO^-_3 N).		
	48 hour EC_{50}	**96 hour LC_{50}**	**[$]NOEC**
Preliminary test	517.73 (95%CI = 441.6 – 1027.7)	683 (95%CI = 361 – 725.8)	n/a*
Definitive test	398.7 (95%CI = 233.6 – 545.8)	781.07 (95%CI = 586.9 – 942.4)	<100

[$]NOEC = No observed effect concentration

*NOEC not applicable as there were affects manifest at lowest exposure concentration.

When the results were compared with toxicity test data for the common toad (Xu, 1997), individual frog larvae exposed to ammonium nitrate, at a minimum of stage 22 of development (Gosner, 1960) were nearly 3 times more sensitive to ammonium nitrate than toad larvae. The toad experiments were carried out using groups of larvae that were fed for the duration of the exposure period. The food

(boiled lettuce) may well have mitigated the less sensitive response of the larvae to the affects of ammonium nitrate. Given the response of the larvae under these experimental conditions, it was thought that frogspawn should be treated in a similar way, to achieve values for EC_{50} and LC_{50}. This was not possible due to the poor replication in the frog spawn test design.

CHAPTER SIX

THE EFFECT OF CHRONIC EXPOSURE TO AMMONIUM NITRATE ON DEVELOPING COMMON FROG (*Rana temporaria*) LARVAE

CHAPTER SIX

THE EFFECT OF CHRONIC EXPOSURE TO AMMONIUM NITRATE ON THE DEVELOPING COMMON FROG '*Rana temporaria*' LARVAE

6.1 INTRODUCTION

Concentrations of nitrate in field ponds were found to persist at relatively high levels for the duration of the common frog breeding season. It was therefore important to determine the long-term effect or chronic impact of exposing developing frog larvae to ammonium nitrate. This was achieved using a flow-through or dynamic test system designed to permit chronic exposure of large numbers of frog larvae to a range of ammonium nitrate concentrations. A range of concentrations were selected that were lower than the EC_{50} concentrations established in Chapter 5.

The effect on physiological development was assessed. This was achieved by exposing larvae from wild collected populations, at an early stage of development, through to front limb emergence before metamorphosis. This was used as a suitable end-point for the investigation in the chronic investigation where individuals were exposed for the duration of the larval period.

Static test systems have the convenience of being easy to use and monitor over short-term investigations, but lack the ability to continually maintain test concentrations. Static systems are most appropriate where test chemicals are stable in solution and maintenance of test concentrations relative to nominal values over the duration of a test is required.

Ammonium nitrate fertiliser is extremely soluble in water, where it becomes hydrated and partially dissociated into component ions.

$$NH_3(gas) + H_2O \rightleftarrows NH_3.H_2O\ (aq) \rightleftarrows NH^+_4\ (aq) + OH^-\ (aq)$$

In solution, NH^{+}_{4} will dissociate from $NH^{+}_{4}NO^{-}_{3}$ and form a weakly alkaline solution with an increase in pH, liberating NH_3. When $NH^{+}_{4}NO^{-}_{3}$ is added to deionised water with a residual pH of between 7.2 and 7.5, the preference is to maintain NH_4^+ levels at approaching slightly acid pH and thereby not liberating NH_3.

Due to the negative charge associated with NO_3^- it becomes quickly bound to any free cations in the water. In a static test system, the concentration of free hydroxyl ions would increase over the duration of the test, producing an alkaline environment, as larvae release excretory waste products of metabolism mainly as ammonia (as they are ammonotelic) into the surrounding media.

To reduce the risk of free NH_3 build up in test solutions, the solutions of fertiliser in the test system were renewed continuously under a dynamic flow through testing regime. This ensured that effects were related to nominal exposure concentrations (i.e. measured concentrations to be within 20% of nominal values) of ammonium nitrate fertiliser (and that they were adequately maintained for the duration of the test) and not because of ammonia toxicity.

In addition, the flow through system continuously removes metabolites and possible bacteria that may build up in the test system. Levels of pH were corrected when each test solution was prepared using either a dilute acid or alkali solution to correct to between 7.0 and 7.5. Dissolved oxygen levels were maintained using the 'cascade' technique, where levels of aeration between test vessels are maintained by the physical action of water passing between test vessels.

6.2 METHODS

6.2.1 Selection of test concentrations for the preliminary flow through test

Three test concentrations and a control were selected for the test. The nominal concentrations were 25, 50, and 100 mg/l NO_3^--N (equivalent to 32.3, 64.5 and 129 mg/l as ammonium nitrate) with a control treatment of artificial pond water alone. The selected concentrations span the range of NO_3^--N concentrations recorded in the field. Similar concentrations of nitrate have been used in research into the effects of nitrate on the common toad using static systems (Baker & Waights, 1993; Xu & Oldham, 1997)).

6.2.2 Flow-through test system design for preliminary investigation one

Preliminary investigations were started in the spring of 1994. The test system was designed and built to expose the highest number of developing common frog larvae to ammonium nitrate solutions at various concentrations, in discrete channels. This was achieved by adhering to the following criteria;

- To deliver test solutions using discrete channels.
- To hold 320 larvae from stage 22 (approx.) through to stage 42 of development.
- To operate continuously and maintain exposure concentrations to within 20% of nominal values.
- To be easily maintained, with easy access and be portable.
- To utilise a mechanical method of aeration included into the design and be able to maintain levels of dissolved oxygen at >60%.

Test solutions were delivered through four discrete channels using a peristaltic pump, (Watson & Marlow 205U attached with a 205BA-cartridge head). Each series contained nine replicate test vessels. Each test vessel was a 500ml plastic 'Azlon' beaker (supplied by BDH Merck). Concentrated stock solution and

dilution water were continuously pumped into the uppermost vessels in each series, providing the required concentrations of test media to each vessel held in series below the mixing vessel.

The appropriate volumes of diluent and toxicant were established using manifold tubing of different bore sizes to achieve each of the required exposure concentrations. A control series of beakers with only dilution water (APW) was also delivered using manifold tubing of an appropriate bore size. A total of 40 test vessels were attached to an upright plywood board (100cm (width) x 150cm (height). The test vessels were secured to the board using plastic coated garden wire passed through holes in the board and looped around the neck of each individual beaker. The wire was secured by twisting at the back of the board. This was repeated for each of the beakers in each series. Within each column, test vessels were staggered above each other, so that the outlet of each test vessel overflowed into the centre of the test vessel on the next row in the series. A single 1cm-diameter hole was drilled into each beaker at a level such that each beaker was full of test solution; it would contain at least 500ml of test media or control solution. On the inside of each beaker, covering the 1cm diameter drilled hole, a small square of Netlon® mesh (square pore size *ca.* 0.5mm) approximately 1 cm square was attached using aquarium sealer. This prevented the loss of larvae from the test vessels. Under the lower edge of each 1cm diameter drilled hole, on the outside of each test vessel, a small spout was formed using aquarium silicon sealer that was initially moulded around a preformed spout made from strong plastic gymnasium line marking tape. The spout was designed to prevent test media running down the side of test vessels and dripping from the underside of each beaker and potentially missing the vessel located below. This aided the 'cascade' aeration of the test media held in the test system. Test media flowed freely between each of the test vessels, dripping at a rate equivalent to 2ml/min (120ml/hour = 2.88 litres / 24 hours).

Plate 6.1 Common frog larval continuous renewal (flow-through) testing apparatus for exposing common frog larvae to ammonium nitrate fertiliser in solution.

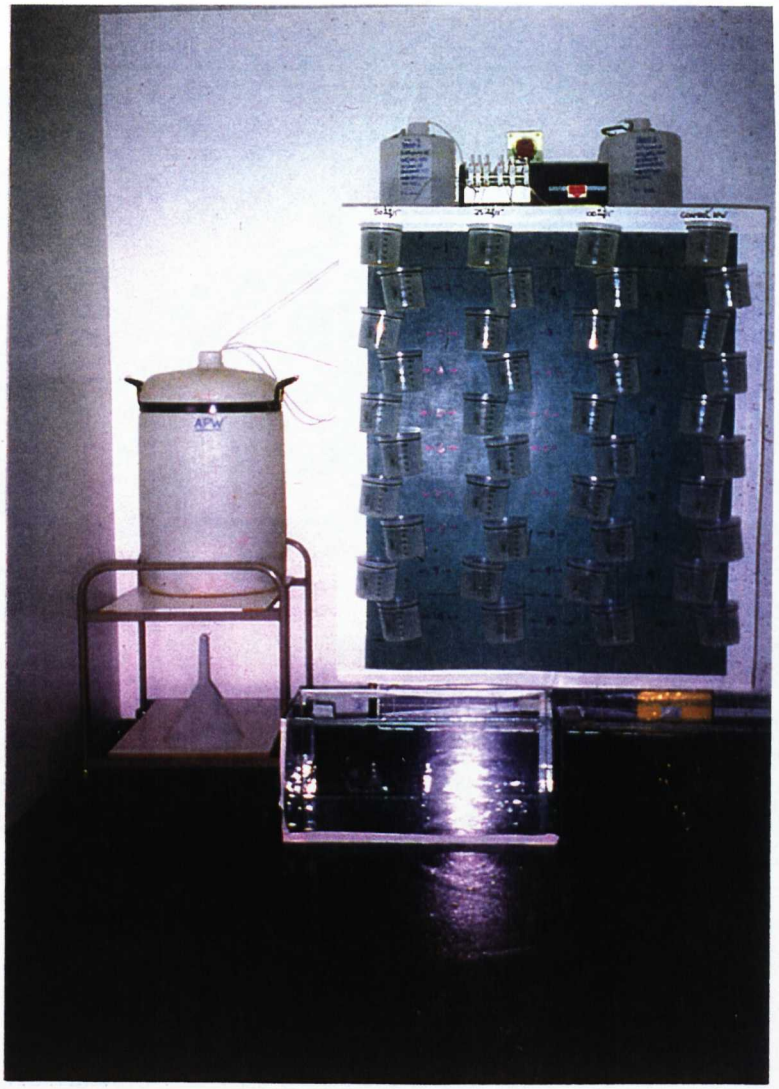


Plate 6.2 Diluent and toxicant aspirators and peristaltic pumping system for the Common frog larval continuous renewal (flow-through) testing apparatus.

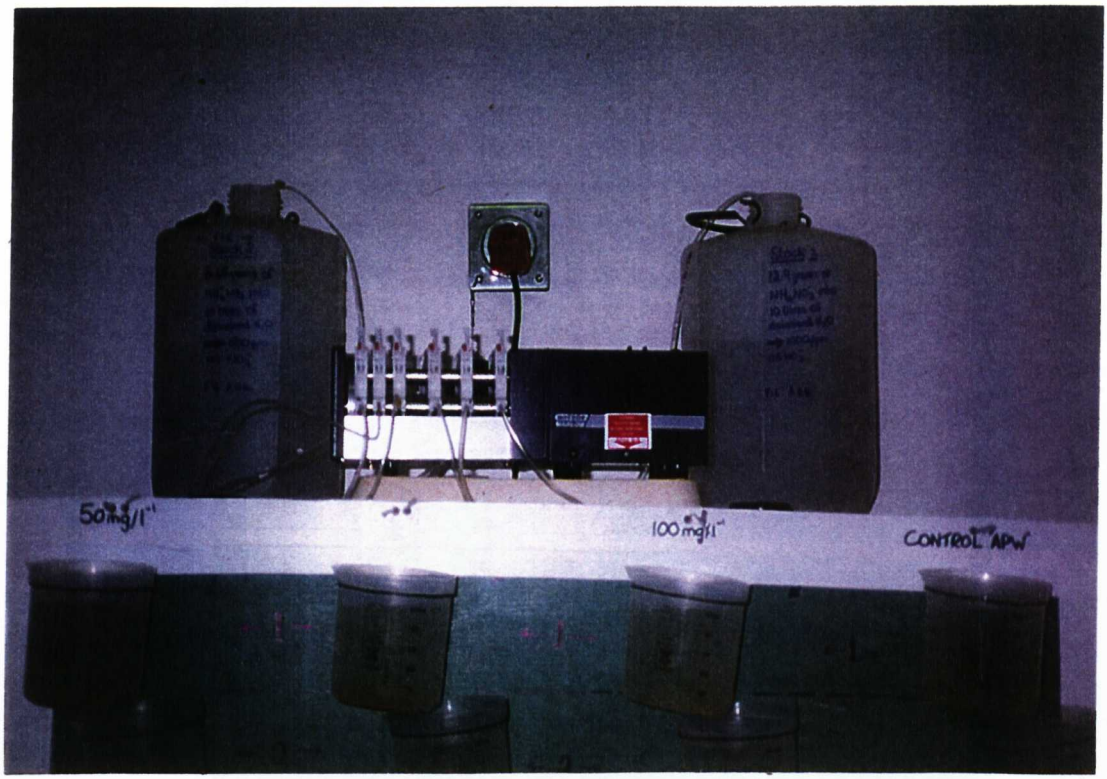


Plate 6.3 Example of the continuous renewal (flow-through) testing vessels held in series on the flow-through testing apparatus, showing the attachment of vessels to support board using wire, spout construction, overflow hole, netting (to prevent loss of larvae from vessel) and staggered attachment to aid aeration via cascade effect.

The board holding the test vessels was attached to a stainless steel cage trolley of approximate dimensions 1.5meters tall x 0.8 meters width x 0.8meters depth. This made the system portable. (Plates 6.1 – 6.3).

6.2.3 Preparation of stock and test solutions

Test solutions were prepared by mixing the appropriate concentrated toxicant stock solution of fertiliser (prepared using artificial pond water and ammonium nitrate) with a known volume of diluent (artificial pond water). This was achieved by pumping concentrated test solution of fertiliser into a mixing vessel (first beaker in each series) which also received a known volume of diluent. As the addition of media to the mixing vessel was continuous, the relative volumes added to the mixing vessels in each series of test vessels dictated the concentration prepared.

Artificial pond water was prepared in advance using laboratory reagent (analytical grade) salts, added directly to reverse osmosis water. The recipe for the preparation of artificial pond water is shown in Table 6.1.

Table 6.1. Ingredients of artificial pond water and preparation details.

Reagent	Amount required (gram per litre) for stock preparation.	Volume (ml) of stock required per litre of APW
$CaCl_2.H_2O$	58.8	5
$MgSO_4.7H_2O$	24.7	5
$NaHCO_3$	12.4	5
KCl	1.15	5

1- litre volumes stock of each salt was prepared as above and then 5ml aliquot of each stock solution was added to reverse osmosis water for every litre of artificial pond water required.

Batches of artificial pond water (80 litres) were prepared and aerated overnight at constant room temperature (*ca.* 17°C ± 2°C) prior to use.

Concentrated stock solutions were prepared at 500 and 1000mg/l NO_3–N using ammonium nitrate fertiliser (Supplied by Chas Draper Farming Supply Merchants, Leicester). Each stock was prepared in a 10-litre polypropylene aspirator by adding ammonium nitrate directly to artificial pond water. Each aspirator was shaken until the fertiliser had dissolved. Test concentrations were prepared using a combination of manifold tubing of various flow rates dependent on required concentration. Each tube had a predetermined flow rate range dependent on the rpm of the pump used and the internal bore diameter of the manifold tubing. Each tube was identified by a unique colour code that corresponded to a manufacturers established flow rate (Watson & Marlow). Two manifold tubes were used to supply each series of test vessels. One for each of the toxicants and the diluent.

The nominal test concentrations 25, 50 & 100 mg/l NO_3^- – N were achieved by calculating the required dilution factors between each of the nominal test concentrations and the nominal concentrations of the stock solutions. Manifold tubing was selected (Watson & Marlow, Peristaltic pump 202S/AA Operating Instructions), to give a dilution factors that would achieve test concentrations within 80% of nominal values. Dilution factors and manifold tubing were calculated as follows:

Dilution factor (x) = stock concentration / required test concentration.

For a nominal test concentration of 25mg/l as NO_3^-– N using a 500mg/l concentrated stock solution; **x = 500 / 25**

x = 20

With a nominal dilution factor of 20, the combination of manifold tubing required to achieve a diluent to toxicant flow rate dilution factor of *ca.* 20 were;

flow rate (ml/min)= 10.2 (purple/white tubing) = artificial pond water (diluent).

flow rate (ml/min)= 0.57 (orange/white tubing) = fertiliser solution (toxicant).

The actual dilution factor between the diluent and toxicant tubing was of 17.89. The proposed concentrated stock solution was then corrected for the NO_3 – N component by multiplying by 1.29 to give the amount of NH_4NO_3 required to obtain the nominal concentration of NO_3^- –N that was required using available manifold tubing.

The same method of calculation was used to establish the tubing combinations required for 50 and 100 mg/l as NO_3^- – N. A nominal dilution factor of 20 was achieved using a 1000mg/l concentrated stock (1000/50 = 20). Therefore, the same tubing combination used to achieve the 25mg/l exposure concentration was used for the 50 mg/l test concentration. For the 100mg/l nominal exposure concentration, 1000 mg/l stock was used giving a nominal dilution factor of 10. The nearest combination of manifold tubing gave a dilution factor of 9.36 (APW flow rate (ml/min) = 10.2 (purple/white); Toxicant flow rate (ml/min) = 1.09 (orange/orange)). Flow rates of the combined toxicant and diluent into the mixing vessels were checked at least weekly when diluent stocks were replenished, with stocks replenished approximately every 5 days. Sufficient concentrated stocks were prepared to last for up to 7 days. With a nominal flow rate of 2ml/min, the nominal daily volume of test media passing through each series of beakers was 2.88 litres. Toxicant concentrated stocks were prepared at 645 and 1290 mg/l as NH_4NO_3. Tubing selection and dilution factors are summarised in Table 6.2.
The tenth row of test vessels (the last vessel in each series) contained no animals and overflowed into waste containers that were emptied daily.

Table 6.2. Manifold tubing selection for the peristaltic pump for delivery of toxicant and diluent to test vessels. (Watson & Marlow, Peristaltic pump 202S/AA; Operating Instructions) showing selection of manifold tubing according to dilution factors between stock and proposed test concentrations.

Test concentration (mg/l NO_3^--N)	Stock concentration (mg/l NO_3^--N)		Required dilution factor	Manifold tubing flow rate (ml/min)		Dilution factor	% nominal
	500	1000					
25	√	X	20	10.2 (a)*	0.57 (b)*	17.89	89
50	x	√	20	10.2 (a)	0.57 (b)	17.89	89
100	x	√	10	10.2 (a)	1.09 (c)*	9.36	94

*(Manifold tubing manufacturers colour coding (a) = Purple/white; (b) = Orange / white; (c) = Orange / orange).

6.2.4 Test vessel larval loading rates.

For any single point in a 24-hour period, the initial larval loading rate was equivalent to the total mass of larvae held in the test vessels, passing through that vessel during a 24-hour period. This accounted for the increasing demand placed on the test system as larvae developed within the test system.

A suitable test vessel volume designed to minimise the effect of animal density was obtained from aquatic toxicity testing regulatory guidelines (OECD document L 383 A, Part C.1. Acute Toxicity for Fish 1992) where recommendations are made as to the suitable loading rates (g) of organism per litre of test media during a test.

The test used animals from approximate larval stage 23 until stage 42 (Gosner, 1960) that is from free-swimming larvae to front limb emergence. The later stages were omitted because individuals tend to reduce feeding rates and lose mass as they absorb stored energy from their tail. Aeration of test media was

supplemented by a constant aeration of the stocks of diluent (artificial pond water).

6.2.5 Larval feeding during the test

A comparative feeding trial was undertaken before the start of the flow-through study. Common frog larvae were fed a diet of ground fish pellets (Tetramin ® floating fish food) ground to a fine powder and added to test vessels on an *ad libatum* basis. Food surplus was siphoned (from the test vessel base) or sieved (from surface of test media) from each test vessel on a daily basis.

To each test vessel was added a 2 to 3 *x* 1cm squares of boiled fresh lettuce (fresh lettuce added to boiling water for 2 minutes) as an additional food source and as a refuge for the duration of the test. This was replaced approximately once every three days over the duration of the test.

6.2.6 Test system pre-conditioning and environmental conditions

The test system was held in an air-conditioned testing facility, with ambient temperatures maintained at 16 ± 2°C for the study duration. Before the addition of larvae to the test system, it was pre-conditioned for approximately 5 days. During this period, the residual nitrate concentrations were measured from randomly selected test vessels in each series of test vessels on a daily basis. This ensured that concentrations were within acceptable limits for the test to start, and that a dynamic equilibrium of concentration existed between test vessels before the start of the test.

6.2.7 Larval body measurements

Larval body, body length (without tail) and total body length (including tail), of 3 arbitrarily selected larvae, were determined by sampling 3 larvae from each test vessel. Individual larvae were carefully removed using a small plastic sieve, and placed on a paper towel. Each larva was transferred to a weighing boat (using a plastic spatula) on a tarred (Mettler 'Toledo' 4 place flat pan balance) balance and

the weights were recorded. A pair of vernier callipers (body length measurements) were used to record the body length measurements whilst the animals were in the petri dish containing media during this procedure to minimise handling stress to the individual larvae. Measurements were made at between 3 and 5 day intervals for the duration of the test. The average mass (mg) and the associated 95% confidence intervals were recorded. The body mass values and associated 95% confidence intervals and analysis are shown in Table 6.6. These were analysed using ANCOVA (analysis of covariance) to establish the effect of vessel replication on the mean body mass and by one way ANOVA, to establish the effect of day number and treatment on each sampling occasion.

Frog larvae were selected from a holding tank that contained 5 litres of aerated artificial pond and 500 larvae (approximately stage 23), into randomly selected test vessels (selected using random number tables). A small plastic sieve was used to transfer 5 individual larvae to each vessel until all vessels from the second row down to the ninth row of each exposure concentration series of test vessels, contained 10 larvae. The start of the test was after the addition of the last larvae to the test vessel.

6.2.8 Procedures during the test

In addition to the removal of excess food (Section 6.2.5), faecal debris from the bottom of each test vessel was siphoned off using a long glass pipette and a length of silicon tubing. Any dead larvae were removed, recording the date, exposure concentration and test vessel number. Notes were made of larval deformities in each series of test vessels, with the number of affected larvae and the type of deformity being recorded. Additional observations were made on larval swimming co-ordination, feeding activity, and colour changes. Body mass and total body length (nose to start of tail) were measured using 3 arbitrarily selected larvae from each test vessel, at weekly intervals for the duration of the test. Concentrations of nitrate in solution held in the test system were monitored approximately at weekly intervals. Water quality parameters were also recorded

at approximately weekly intervals. These comprised pH, dissolved oxygen and water hardness (recorded for each new batch of artificial pond water).

6.2.9 Statistical analysis of results

The percent hatching, survival rates and malformations were transformed to logit values and arc-sine transformations. This was necessary, as percentages (proportions) tend not to follow a normal distribution. The mean body masses and total body lengths were compared between treatments using analysis of variance. Linear regression was used to compare the impact of treatment on growth rates.

6.3 RESULTS OF INVESTIGATION ONE

6.3.1 Larval mortality

The initial test was abandoned after 25 days following high mortality rates in control test vessels (> 20% on day 14 (39% cumulative mortality, with 68% cumulative control mortality by day 25 of the test)). Body length, body mass, and total body length were recorded weekly up until day 23.

High rates of mortality were observed in all test concentrations, with significant mortality at the highest concentrations (Table 6.3). Mortality rates increased with concentration of fertiliser. At 25 mg/l NO_3^--N, by day 13, there was a cumulative percentage mortality of 69% increasing to 81% by day 16 and 84% by day 25. At 50 mg/l NO_3^--N, by day 11, cumulative percentage mortality was 66%, increasing to 100% mortality by day 16. At the highest concentration of 100mg/l NO_3^--N, by day 8, cumulative percentage mortality was 100%. Survival was assessed at the end of the first test with a dose response curve shown in Figure 6.1. Similar stages of development were achieved at all concentrations in all surviving larvae on day 25.

However, these results are ambiguous, as the episode of high mortality may have been attributable to a bacterial infection across all test vessels, was observed and may have influenced the graded mortality across exposure concentrations.

Figure 6.1. Dose-response curve showing percentage mortality rates for the first larval flow through investigation. Control (◊), 25mg/l (□), 50 mg/l (Δ) and 100 mg/l NO_3-N (x).

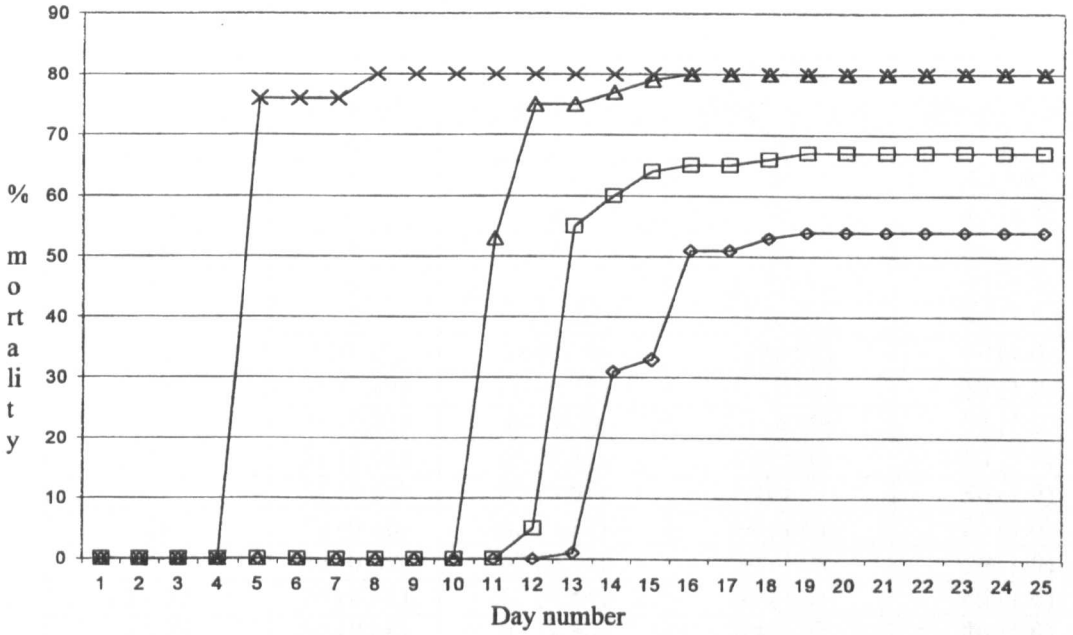


Table 6.3. Cumulative mortality results from flow-through Investigation One, showing the proportion of dead larvae (in brackets) relative to the starting number (80) of larvae at each concentration.

Day number	Number of dead larvae			
	Control	25mg/l NO^-_3-N	50mg/l NO^-_3-N	100mg/l NO^-_3-N
0-5	0	0	0	0
5-7	0	0	0	76 (0.95)
8-9	0	0	0	80 (1.0)
10	0	0	0	80 (1.0)
11	0	0	53 (0.66)	80 (1.0)
12	0	5 (0.06)	75 (0.94)	80 (1.0)
13	1 (0.01)	55 (0.69)	75 (0.94)	80 (1.0)
14	31 (0.39)	60 (0.75)	77 (0.96)	80 (1.0)
15	33 (0.41)	64 (0.8)	79 (0.99)	80 (1.0)
16	51 (0.64)	65 (0.81)	80 (1.0)	80 (1.0)
17	51 (0.64)	65 (0.81)	80 (1.0)	80 (1.0)
18	53 (0.66)	66 (0.83)	80 (1.0)	80 (1.0)
19	54 (0.68)	67 (0.84)	80 (1.0)	80 (1.0)
20-22	54 (0.68)	67 (0.84)	80 (1.0)	80 (1.0)
23-25	54 (0.68)	67 (0.84)	80 (1.0)	80 (1.0)

6.3.2 Body mass and total body length measurements

Mass and length measurements were continued until day 19 of the test, with five measurements before the test was abandoned. On each occasion, 3 randomly selected larvae were weighed and measured from each vessel. This data are summarised in Table 6.4, and displayed in Figures 6.2 and 6.3.

Larval total body length.

Larval total body mass results for investigation 1 are summarised in Table 6.4.

At the start of the test, larval mean total body lengths (Table 6.4) were not significantly different ($F=1.95$; $p=0.144$). Over the duration of investigation one, concentration of fertiliser did have a significant effect on the total larval body

Figure 6.2 Larval total body length (mm) against Day number for flow through Investigation One (1 SD shown).

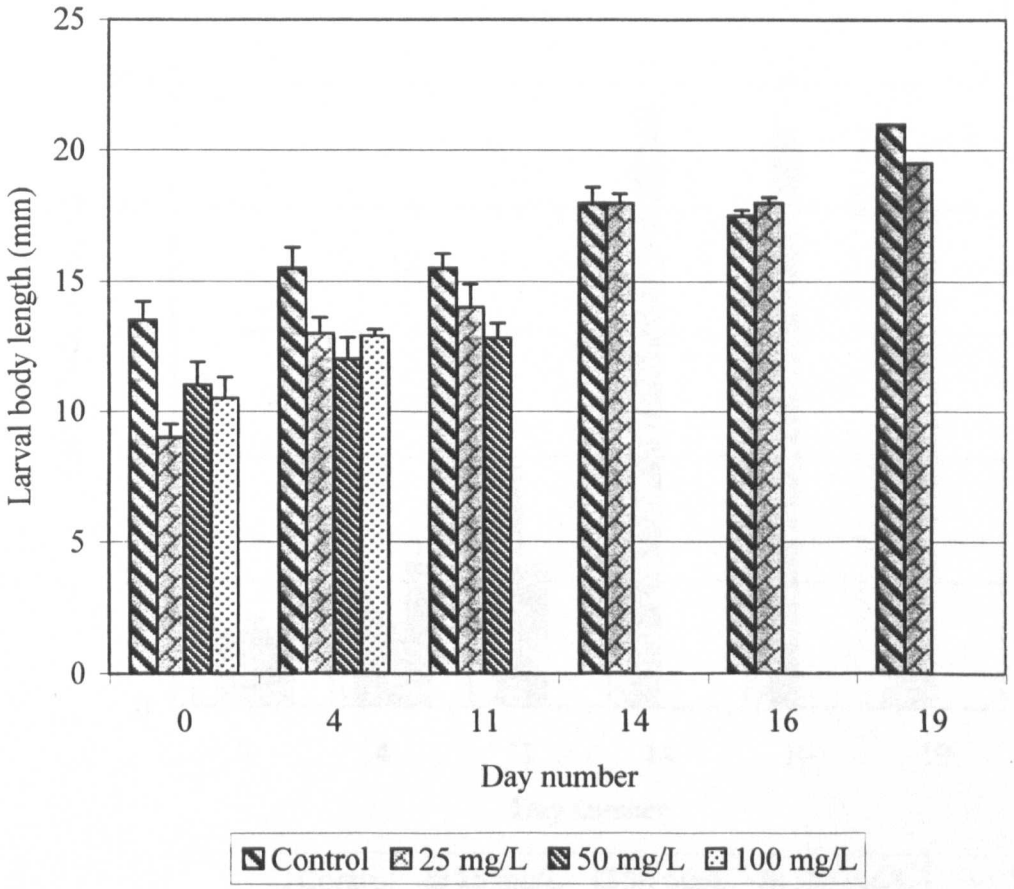
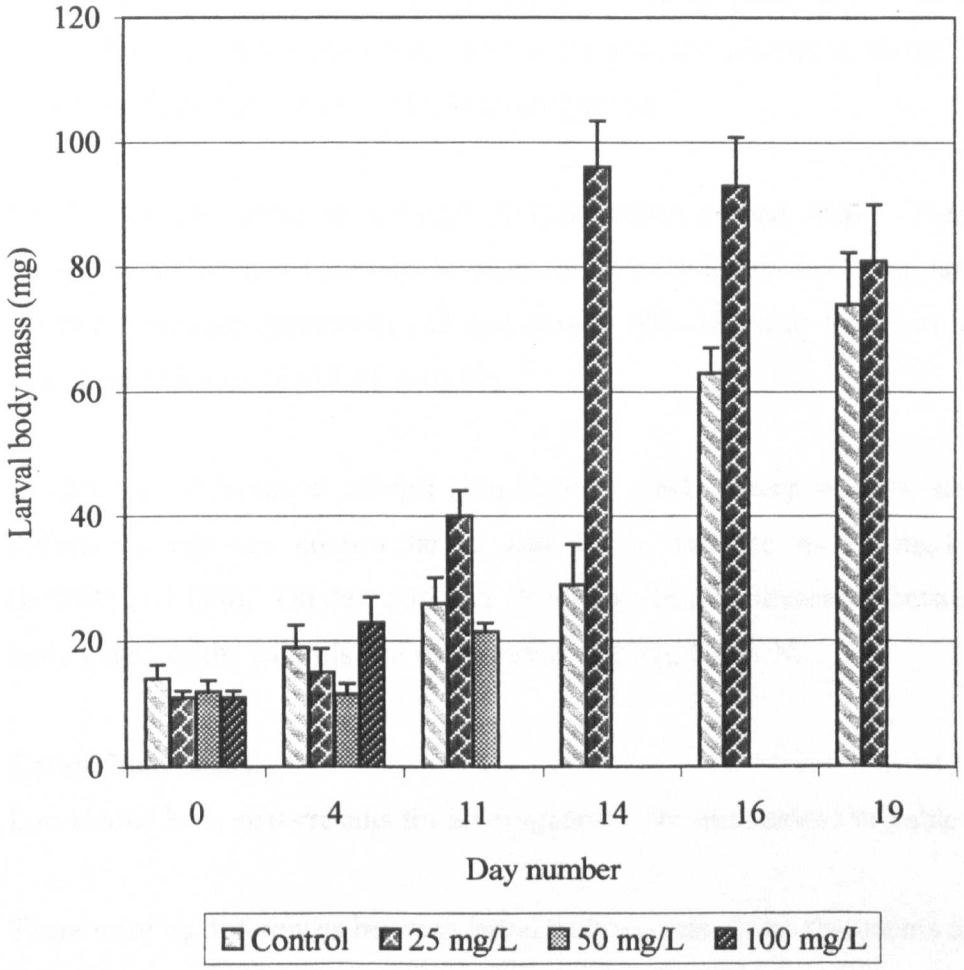


Figure 6.3 Larval body mass (mg) against Day number for flow through Investigation One (showing 1 S.D).



length (F=18.90; p<0.01), with body lengths decreasing with increasing concentration of ammonium nitrate.

At day 4, 96 hours into the test, there were significant difference in mean body lengths (F=-21.69; p<0.01), with treated larvae being significantly less than the control larvae. Larval body lengths were significantly shorter at 50mg/l NO_3-N, than control, 25 and 100mg/l NO_3-N treated larvae.

By day 11 all larvae at 100mg/l NO_3-N treatment had died. There were significant differences between the mean larval body length in control larvae and the two remaining treatments (25 and 50mg/l NO_3-N), with the controls being significantly longer (F=19.41; p<0.01).

By day 14, all larvae at 50mg/l NO_3-N were dead. There were no detectable differences between control larvae and larvae exposed at 25 mg/l NO_3-N (F=0.00; p=1.000). On day's 16 and 19 no significant differences between total body length of the controls and those larvae at 25mg/l NO_3-N.

Larval body mass.

Larval total body mass results for investigation 1 are summarised in Table 6.4.

There were no differences between larval body masses across treatments on Day 0 (one-way anova: F=1.57; p=0.218). Exposure concentration was found to have a significant effect on total larval body mass relative to the control (F=18.51; p<0.01) over the duration of the test.

By day 4, there was a significant difference in the body masses between treatments (F=-18.45; p<0.01), with mean body mass at 50mg/l NO_3-N being significantly less than the controls. No differences were seen between 25 and 100 mg/l NO_3-N treatments and the controls. By day 11, the majority of the 50 and 100mg/l treatment larvae were dead, leading to large confidence intervals due to

lack of data at these treatments. No significant differences existed between the two high doses and the controls. However, at 25mg/l NO_3-N, there were significant differences in mean body masses relative to the controls, with mean larval body mass being greater than the controls. This was repeated on days 14 and 16, until on day 19, control body masses were not significantly different to the 25mg/l treatment ($F=1.23$, $p=0.287$).

Table 6.4. Mass and length measurements for flow-through Investigation One, showing the mean total body length (mm), and the mean body mass (mg) results ± 1 standard error.

Larval body mass (mm) ± 1 standard error				
Day Number	Nitrate concentration (mg/l NO_3-N)			
	Control	25	50	100
0	13.2 ± 4.43	11.1 ± 1.24	11.5 ± 2.02	10.6 ± 1.08
4	18.9 ± 3.66	15.8 ± 3.85	11.4 ± 1.85	23.3 ± 3.45
11	25.5 ± 6.07	39.8 ± 3.49	21.0 ± 1.41	-
14	29.1± 11.1	95.9 ± 6.45	-	-
16	64.1 ± 6.81	92.1 ± 8.34	-	-
19	74.3± 9.97	80.1 ± 11.2	-	-
Larval total body length (mm)) ± 1 standard error				
0	11.4 ± 1.13	9.75 ± 1.20	10.9 ± 1.60	10.4 ± 1.59
4	15.6 ± 0.92	13.4 ± 0.52	11.2 ± 1.85	12.9 ± 0.64
11	15.8 ± 0.84	14.4 ± 0.52	12.8 ± 0.35	-
14	18.8 ± 0.89	18.8 ± 2.71	-	-
16	18.6 ± 2.00	19.8 ± 2.38	-	-
19	21.1 ± 2.03	20.3 ± 1.31	-	-

6.4 Flow-through Investigation Two: Repeat flow through test using frog larvae

Due to poor test system maintenance and poor levels of survival, the flow-through investigation was repeated. In the repeat study, better levels of maintenance was achieved by siphoning or sieving waste material from each test vessel at least three times in any seven-day period.

Before the test re-start, the test system was dismantled and cleaned using an anti-bacterial (non- residue) decontaminating detergent (Decon 90®).

All vessel outlet spouts and the Netlon ® squares that covered the outlet holes were replaced. Fresh manifold tubing and clean fertiliser stock aspirators were used. The system was reassembled and the pumps were restarted under test conditions for seven days using deionised water only. Following the acclimation period, fertiliser test solutions were prepared and the test system was primed in the same way as for Investigation One.

6.4.1 Larval selection for flow-through Investigation Two

The same methods of larval selection employed for Investigation One were used for the repeat study. Rural ponds in the surrounding countryside were again inspected for common frog larvae at an early stage of development. Larvae were collected from Quenby New Pond (SK 704061) where the residual concentrations of NO_3^--N were < 3 mg/l, and suitable developing larvae were present (still held in distinct spawn clumps, and most were at stage 18-20 of development). Four small spawn clumps collected with >1000 developing larvae. These were returned to the laboratory in pond water collected from the site and acclimated to test conditions until they had reached stage 22 of their development. Larvae were transferred to a 30-litre all glass aquarium containing 20 litres of acclimated artificial pond water, and allowed to acclimate to test facility conditions for at least 48 hours before being added to the test system.

6.5 RESULTS OF INVESTIGATION TWO

Repeat flow through test system: Investigation Two

6.5.1 Larval Mortality

Low levels of mortality were expected in the exposure concentrations, as the initial LC_{50} was substantially higher than the highest exposure concentration for the flow-through test (100mg/L). Similarly the EC_{50} value (399 mg/L NO_3^--N), was approximately 4 times higher than the highest concentration used in the flow through system. Table 6.5 shows mortality data during the second flow-through

investigation. These results are displayed in Figure 6.4a showing levels of mortality at the end of the biometrics-measuring period and at the point when the last surviving metamorph had emerged from the test system. The cumulative levels of mortality are shown in Figure 6.4b

The number of dead larvae on day 77 and day 96 were transformed (arc-sine transformation) to normalise the data. The data were then analysed using analysis of variance, to assess the relative impacts on the levels of mortality between treatments.

Exposure time had a significant effect on larval mortality across treatments. From the data, it was apparent that the period between the onset of metamorphosis until the point at which the larvae emerged from the test system resulted in a graded increase in mortality with increasing exposure concentration. An analysis of variance detected a significant difference between treatments ($p=0.034$ $df=3$) at the 0.05 level of significance.

Figure 6.4a. Larval mortality on Day 77 (*light shaded bars*); the end the biometric measurement recording period and on Day 96 (*dark shaded bars*) when all surviving larvae had emerged from the test system.

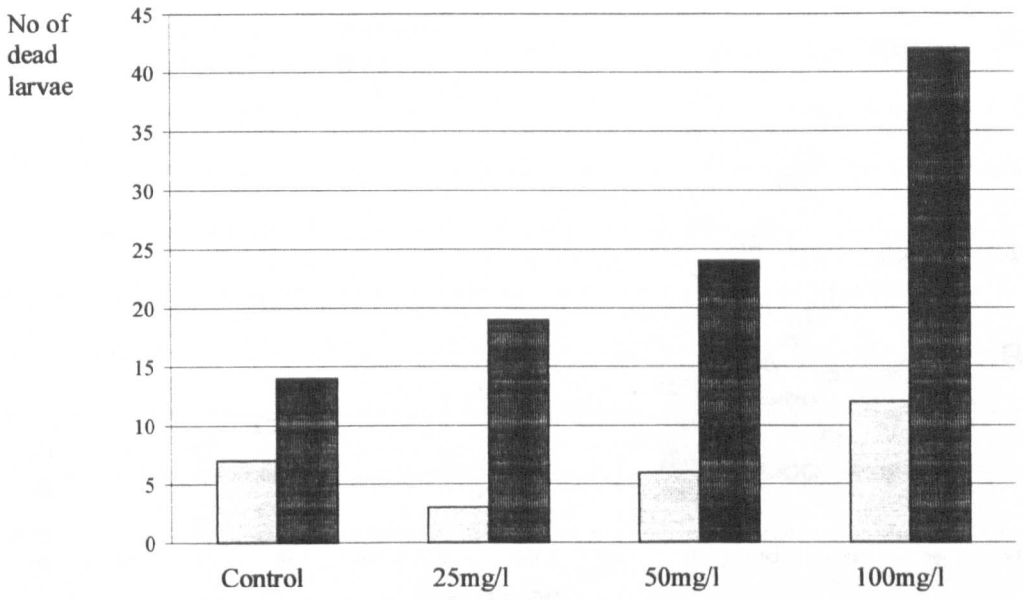


Figure 6.4b Cumulative larval mortality during investigation two for frog larvae exposed under flow-through test conditions to control, 25, 50 and 100mg/l NO_3-N solutions (%mortality on y-axis).

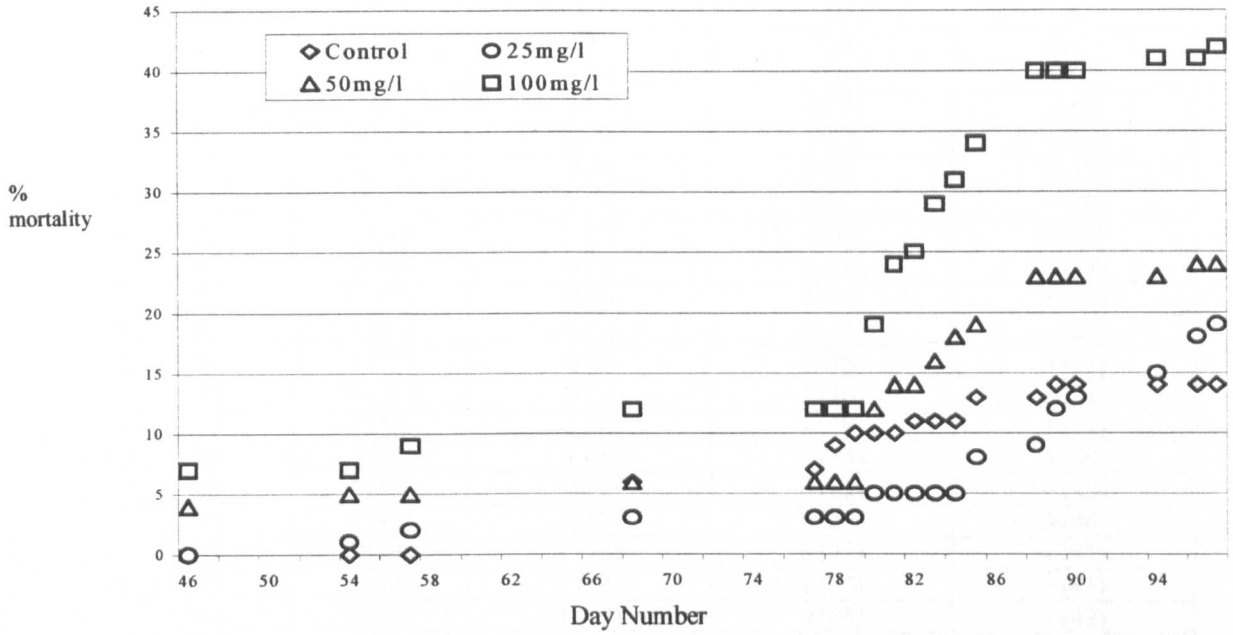


Table 6.5 Larval mortality (cumulative mortality in brackets) during Investigation two (77* indicates the first larvae to reach stage 42 of larval development – front limb bud emergence). % mortality shown.

Day number	Number of dead larvae (cumulative number dead in brackets)			
	Control	25mg/l NO_3^--N	50mg/l NO_3^--N	100mg/l NO_3^--N
0-45	0	0	0	0
46	0	0	4 (4)	7 (7)
54	0	1 (1)	1 (5)	0 (7)
57	0	1 (2)	0 (5)	2 (9)
68	6 (6)	1 (3)	1 (6)	3 (12)
77*	1 (7)	0 (3)	0 (6)	0 (12)
78	2 (9)	0 (3)	0 (6)	0 (12)
79	1 (10)	0 (3)	0 (6)	0 (12)
80	0 (10)	2 (5)	6 (12)	7 (19)
81	0 (10)	0 (5)	2 (14)	5 (24)
82	1 (11)	0 (5)	0 (14)	1 (25)
83	0 (11)	0 (5)	2 (16)	4 (29)
84	0 (11)	0 (5)	2 (18)	2 (31)
85	2 (13)	3 (8)	1 (19)	3 (34)
88	0 (13)	1 (9)	4 (23)	6 (40)
89	1 (14)	3 (12)	0 (23)	0 (40)
90	0 (14)	1 (13)	0 (23)	0 (40)
93	0 (14)	2 (15)	0 (23)	1 (41)
95	0 (14)	3 (18)	1 (24)	0 (41)
96	0 (14)	1 (19)	0 (24)	1 (42)

% Mortality	(14/90) 15.6%	(19/90) 21%	(24/90) 27%	(42/90) 47%

The level of mortality in control test vessels doubled in the 19 day period between day 77 and the end of the test. At the highest concentration, the number of dead larvae had increased almost 4 times over the same period. Levels of mortality were low until day 77 (<15% across all exposure concentrations). During the period of transition (between day 77 and day 96) when larvae were undergoing gross anatomical changes, frog larvae were more susceptible to the effects of the increasing concentrations of ammonium nitrate.

6.5.2 Larval body mass results

The results of body mass measurements in Investigation two are presented in Table 6.6, and presented in Figures 6.5 – 6.8

At the start of the test, it was noted that larvae exposed at 25mg/L NO_3^--N had a significantly greater body mass than larvae exposed at the remaining test concentrations. The variation in the starting body mass may only be explained by sampling variation. During the initial stages of the test, the larvae are developing exponentially, and by day 6, the control larvae have the greatest mean body mass.

The critical phases during larval development occurred during the exponential growth phase at the start of the test, until approximately day 20. During this time, masses of control larvae, were significantly higher than those exposed to fertiliser. Lower mean body masses were recorded at the highest exposure concentration during the first 16 days of the test. On day 16, larvae at 50mg/l record the lowest body mass with the greatest mass recorded at 25mg/l NO_3^--N exposure concentration. From day 23 until day 51, no significant differences between treatments and the control were detected. This represented the plateau phase in the larval growth curve. By day 51, larval body mass was significantly lower in control larvae than in exposed larvae. Body mass at 50mg/l NO_3-N on day 70 was significantly higher than controls, and the remaining exposure concentrations. All Larvae at 50mg/l NO_3^--N had either died or emerged by day 73. By day 77, those larvae exposed at 25 mg/l NO_3^--N were significantly heavier than other larvae in the test system.

The impact of test vessel replicate was also analysed using analysis of covariance, using for each sampling timepoint with vessel replicate being the covariant for the analysis. The effect of replicate on the mean body mass was not significant suggesting that the study design was vertically robust, with no effect of test vessel location within each concentration series of vessels in the testing rig being detected.

Figure 6.5 Control mean larval body mass results (mg) with 95% confidence intervals.

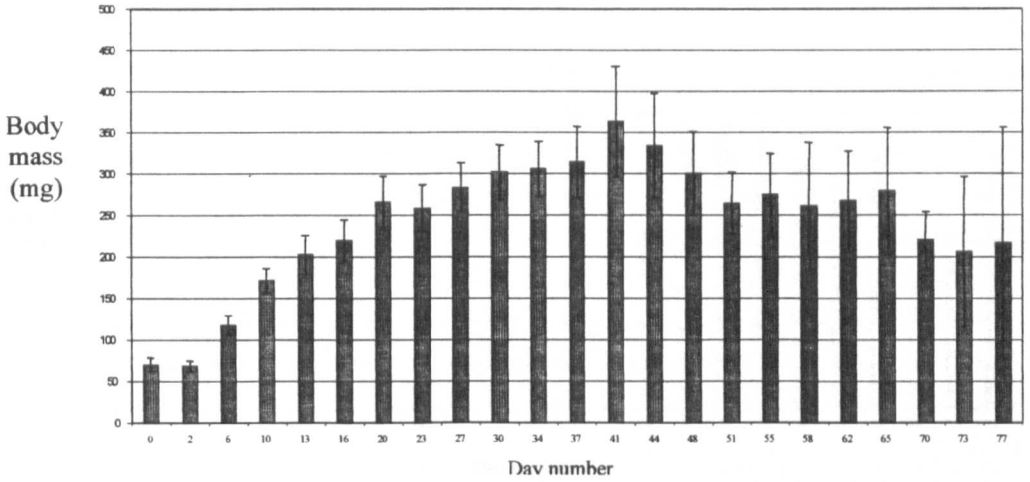


Figure 6.6 Mean larval body mass (mm) for larvae at 25mg/l NO_3-Nwith 95% confidence intervals (y-axis = body mass; x axis = day number).

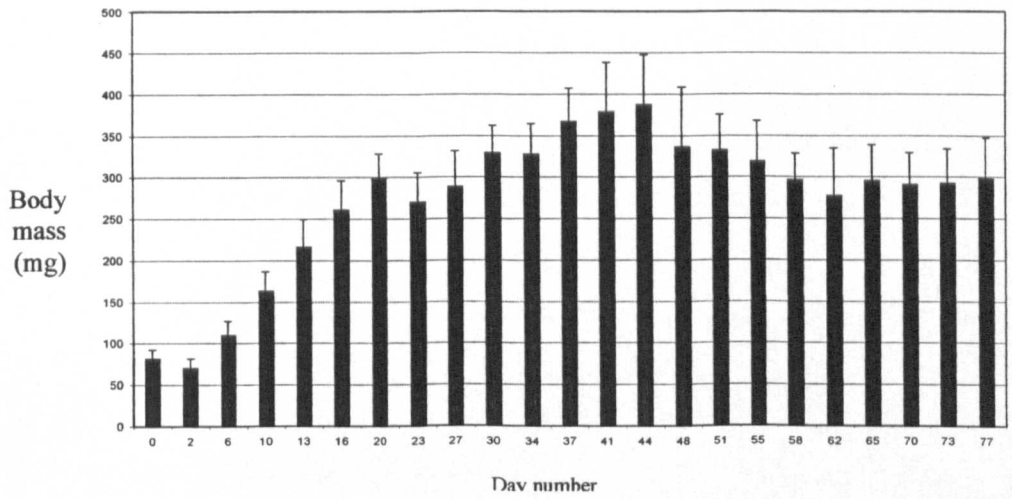


Figure 6.7 Mean larval body mass (mm) for larvae exposed at 50mg/l NO_3-N with 95% confidence intervals (y-axis = body mass; x axis = day number).

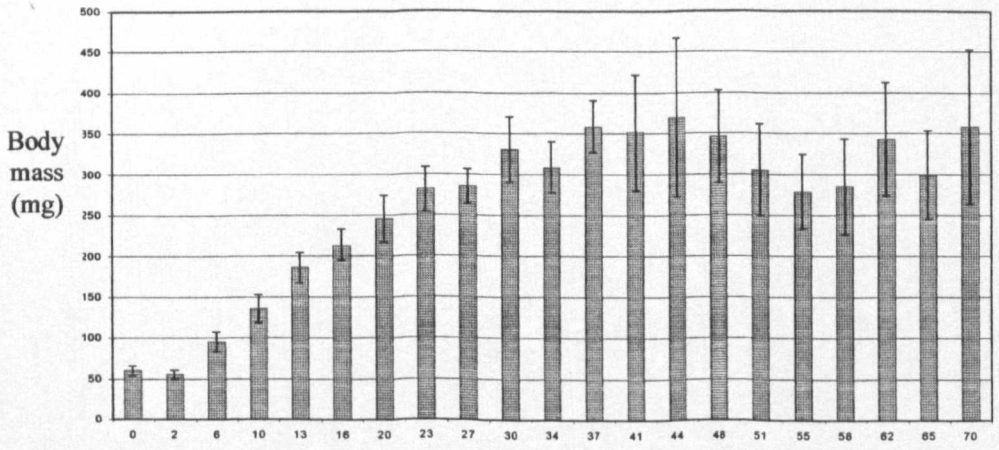


Figure 6.8. Mean larval body mass (mm) for larvae exposed at 100mg/l NO_3-N with 95% confidence intervals (y-axis = body mass; x axis = day number).

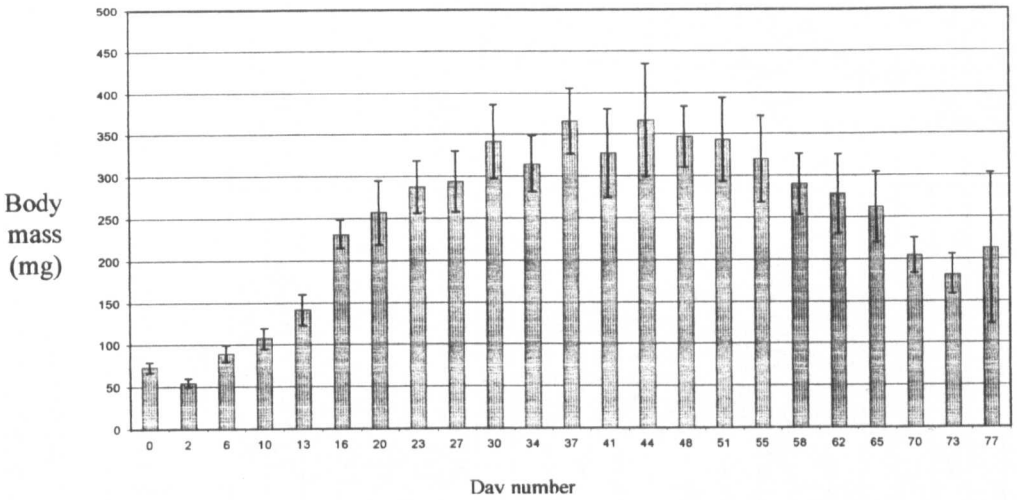


Table 6.6 Mean larval body mass (mg) recorded for developing larvae from stage 22 through to stage 42 (Gosner;1960) for each fertiliser exposure concentration. Also shown are the 95% confidence intervals with the results of analysis of covariance with the levels of significance ($p<0.05$). Where no level is stated, no significant differences between treatments were detected ($p>0.10$). The p value level of significance for the effect of vessel replicate on the body mass is shown in brackets alongside each mean value where significant differences between treatments are detected (df = 7, 1343).

Day number	$Levels of significant difference between treatments	Larval body mass (mg) with 95% confidence intervals for each nitrate (NO_3^--N) exposure concentration.			
		Control	25mg/l	50mg/l	100mg/l
0	P=0.001; F=5.68; 3,92 £df ***	70.5 ± 7.77 (0.583)	82 ± 10.5 (0.989)	60.1 ± 6 (0.890)	73.2 ± 6.29 (0.898)
2	P=0.001; F=5.85; 3,92 df ***	68.3 ± 6.08 (0.562)	70.6 ± 10.9 (0.970)	55.5 ± 5.4 (0.892)	54.6 ± 4.9 (0.698)
6	P<0.001; F=4.33; 3,92 df ***	117.8 ± 11.5 (0.493)	110.3 ± 16.9 (0.778)	95.5 ± 12.3 (0.077)	89.8 ± 9.63 (0.888)
10	P<0.001; F=12.15; 3,92 df ***	171.7 ± 14.4 (0.092)	163.1 ± 23.4 (0.097)	136.1 ± 16.5 (0.101)	107.6 ± 12.5 (0.058)
13	P<0.001; F=7.87; 3,92 df ***	202.8 ± 23.2 (0.247)	216.3 ± 33.2 (0.066)	186.3 ± 18.4 (0.056)	141.5 ± 17.9 (0.124)
16	P=0.029; F=3.15; 3,88 df ***	219.9 ± 24.9 (0.657)	261.7 ± 35 (0.067)	213.8 ± 19.4 (0.087)	231.9 ± 16.1 (0.215)
20	P=0.102; F=2.14; 3,76 df *	266.3 ± 30.96 (0.587)	299.6 ± 29.7 (0.053)	245.9 ± 28.5 (0.065)	257.1 ± 37.6 (0.457)
23	-	258.7 ± 28.21	271.2 ± 34.3	281.7 ± 27.5	287.4 ± 32
27	-	283.9 ± 29.06	289.4 ± 42.2	285.2 ± 21.5	293.9 ± 40
30	-	301 ± 33.4	330 ± 32	330.7 ± 40.2	341.9 ± 43.9
34	-	306.3 ± 32.9	329.3 ± 35	308.8 ± 30.9	315.2 ± 33.8
37	-	314.4 ± 43.1	368.2 ± 39.5	358.6 ± 31.7	366.7 ± 39.3
41	-	363.6 ± 67	379.9 ± 58.9	351.3 ± 70.6	328 ± 52.8
44	-	334.8 ± 63.1	388 ± 60	369.6 ± 97.2	367.4 ± 67.1
48	-	300.9 ± 50.1	337.6 ± 71.8	346 ± 56.7	347 ± 36.5
51	P=0.04; F=3.15; 3,28 df ***	264.9 ± 36.7 (0.064)	333.5 ± 42.1 (0.655)	305.5 ± 56 (0.587)	343.3 ± 50.2 (0.633)
55	-	275.1 ± 49.9	320.1 ± 48.1	279.3 ± 45.7	320.6 ± 51.7
58	-	261.8 ± 76.4	297.1 ± 32.0	285 ± 58.0	290.1 ± 36.6
62	-	267.4 ± 59.9	278.6 ± 56.6	343.3 ± 69.1	278.5 ± 47.2
65	-	279.7 ± 76.3	296 ± 42.6	299.2 ± 54.1	262.8 ± 41.7
70	P<0.001; F=20.62; 3,21 df ***	220.8 ± 33.4 (0.687)	292.2 ± 37.6 (0.939)	358.3 ± 94.2 (0.078)	204.6 ± 20.7 (0.099)
73	P=0.003; F=10.66; 2,10 df ***	206 ± 90.2 (0.684)	293 ± 41.3 (0.984)	-	181.8 ± 23.9 (0.235)
77	P=0.031; F=5.51; 2,8 df ***	217.3 ± 139.6 (0.982)	298.8 ± 48.2 (0.855)	-	213.3 ± 89.7 (0.564)

$ One-way analysis of variance: p values ***<0.05 considered significant. Values *>0.05 &<0.1 were considered to reflect trends .

£ df = degrees of freedom (n-1).

6.5.3 Larval body and total body length results

The mean values with associated 95% confidence intervals are presented in Tables 6.7 and Table 6.8, with the results displayed in Figures 6.9 – 6.16.

Mean body lengths

On day 6, control larvae and those exposed to 25mg/l NO_3^--N were significantly longer, than larvae at 50 and 100mg/l NO_3^--N concentrations. On day's 10 and 13, larvae in the controls, 25mg/l and 50mg/l NO_3^--N concentrations were significantly longer than larvae at 100mg/l NO_3^--N.

Between day's 16 and 65, no differences were detected between the lengths of the controls and the treated tadpoles. On day 70, control larvae were significantly smaller than those exposed to 50mg/L nitrate.

Mean total body lengths

By day 13, larvae at the highest exposure concentration were significantly shorter than larvae in controls, 25, and 50 mg/l NO_3^--N. By the end of the monitoring period, no substantial differences were recorded between the treatment groups.

There was no impact of test vessel replicate on either the body length or total body length during the study at those times when significant differences were detected between treatments.

Table 6.7. Mean total body length (mm) recorded for larvae from stage 22 through to stage 42 (Gosner;1960) for each fertiliser exposure concentration in flow-through Investigation Two. Also shown are the 95% confidence intervals and the results of analysis of covariance with the levels of significance ($p<0.05$). Where no level is stated, values for $p>0.10$ and no significant differences were detected. The p value level of significance for the effect of vessel replicate on the mean total body length (mm) is shown in brackets alongside each mean value where significant differences between treatments are detected (df = 7, 1343).

Day number	$Levels of significant difference between treatments	Total body length (mm) with 95% confidence intervals for each nitrate (NO_3^--N) exposure concentration.			
		Control	25mg/l	50mg/l	100mg/l
0	p=0.019; F=3.46; 3,92 £df***	17.8 ± 0.56 (0.939)	17.4 ± 0.43 (0.330)	16.9 ± 0.38 (0.370)	16.9 ± 0.38 (0.479)
2	-	18±0.52	18 ± 0.90	18 ± 0.53	17.9 ± 0.64
6	p=0.016; F=3.62; 3,92 df**	22.1±0.82 (0.878)	21.7 ± 1.55 (0.576)	20.3 ± 0.93 (0.554)	20.1 ± 0.85 (0.056)
10	P<0.001; F=15.04; 3,92 df***	27.2±0.97 (0.443)	26.6 ± 1.75 (0.224)	24.3 ± 1.22 (0.078)	21.9 ± 1.10 (0.157)
13	P<0.001; F=25.97; 3,92 df***	28.9±1.36 (0.125)	28.9 ± 1.83 (0.222)	29.2 ± 1.22 (0.056)	22.4 ± 1.19 (0.667)
16	-	30.6±1.08	31.3 ± 1.60	30.4 ± 1.24	29.5 ± 1.48
20	-	32.6±1.81	34.1 ± 1.58	32.8 ± 2.11	30.3 ± 2.45
23	-	33.4±1.76	34.8 ± 1.89	34.7 ± 1.96	32.5 ± 1.64
27	-	33.2±1.91	33.5 ± 2.33	34 ± 1.47	34.5 ± 1.79
30	-	34±1.62	35.5 ± 1.37	34.6 ± 2.25	33.9 ± 1.79
34	-	33.3±1.95	34.6 ± 1.96	34.1 ± 1.51	33.1 ± 2.05
37	-	33.4±1.29	34.1 ± 1.51	33.9 ± 1.75	33.8 ± 1.89
41	-	35.5±2.76	35.3 ± 1.71	36.9 ± 2.84	34.8 ± 2.13
44	-	35.5±2.68	38 ± 3.19	35.6 ± 5.86	38.5 ± 2.68
48	-	32.6±2.35	35.4 ± 3.9	35 ± 2.83	34.1 ± 1.92
51	-	32.5±2	33.8 ± 2.08	33.8 ± 3.76	34.5 ± 2.57
55	-	34.9±2.95	33.8 ± 3.45	34.9 ± 3.93	32.8 ± 2.22
58	-	35±3.81	35 ± 3.38	34.3 ± 2.17	34 ± 2.82
62	-	33.4±1.92	33.9 ± 1.64	35.7 ± 2.18	33.6 ± 2.53
65	-	31±0.58	33.2 ± 1.17	33 ± 1.32	31.4 ± 1.10
70	-	32.7±2.09	32.7 ± 3.10	32.7 ± 8.72	30.1 ± 1.86
73	P=0.100; F=2.93; 2,10 df*	30.5±2.75 (0.082)	31.6 ± 1.42 (0.329)	-	29.3 ± 2.39 (0.868)
77	-	30.3±3.8	31.8 ± 2.72	-	29.3 ± 10.8

$ One-way analysis of variance: p values ***<0.05 considered significant. Values *>0.05 &<0.1 were considered to reflect trends .

£ df = degrees of freedom (n-1).

Figure 6.9. Mean total body length (mm) for control larvae with 95% confidence intervals, for flow-through Investigation Two.

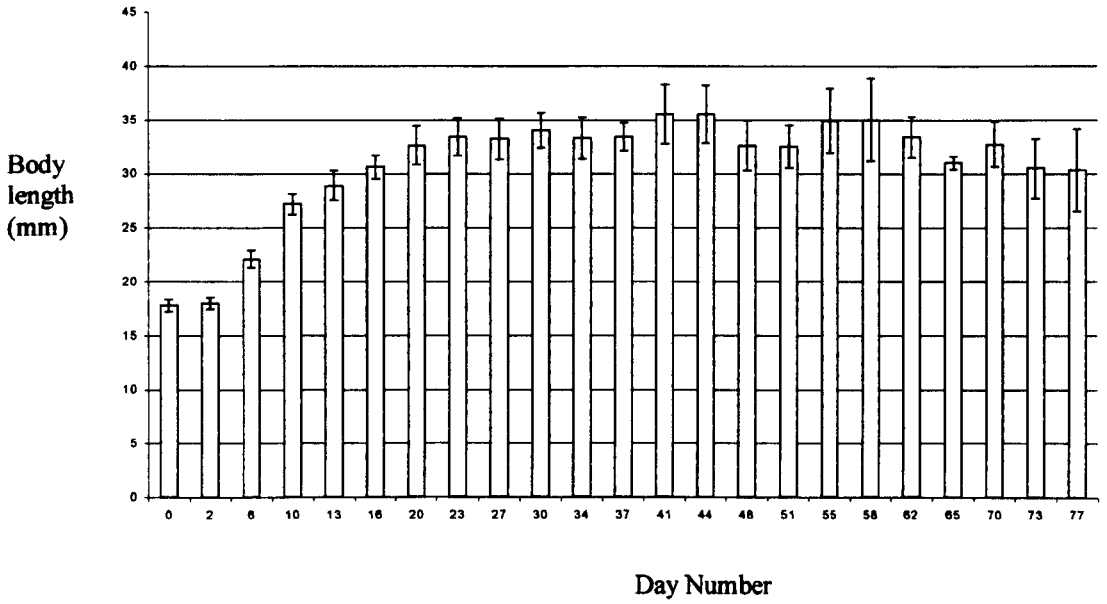


Figure 6.10. Mean total body length (mm) for 25mg/l NO_3-N treated larvae with 95% confidence intervals, for flow-through Investigation Two.

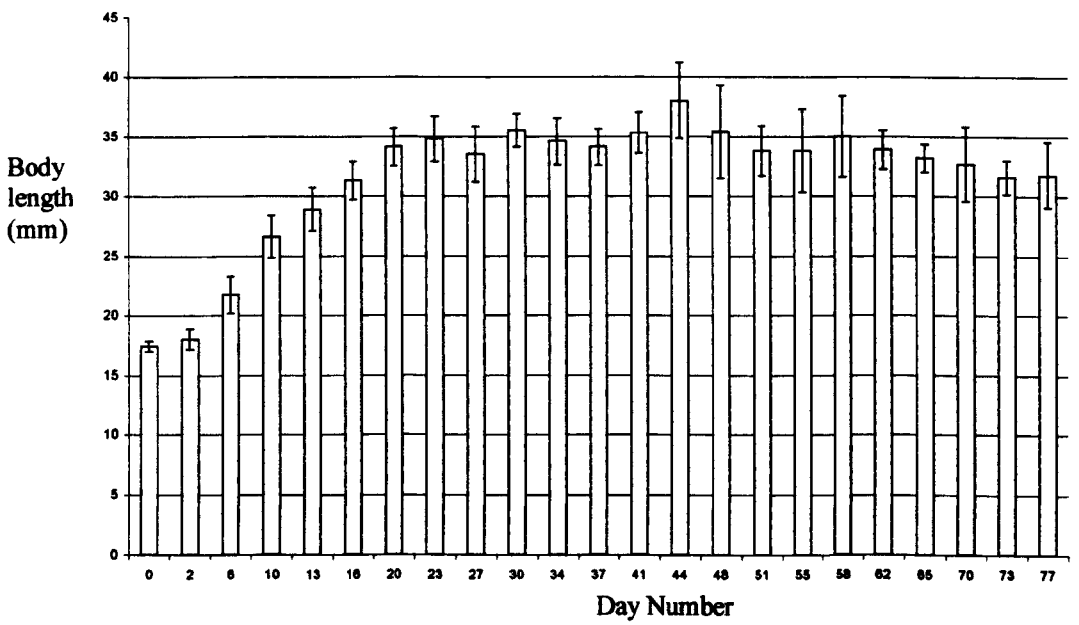


Figure 6.11. Mean total body length (mm) for 50mg/l NO_3-N treated larvae with 95% confidence intervals, for flow-through Investigation Two.

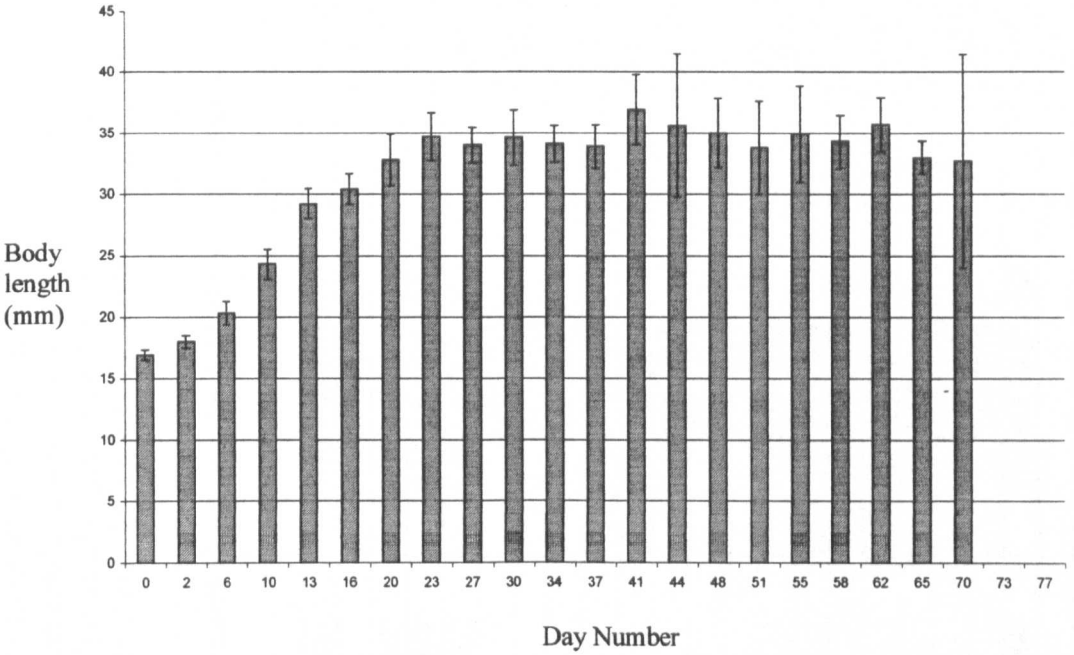


Figure 6.12. Mean total body length (mm) for 100mg/l NO_3-N treated larvae with 95% confidence intervals, for flow-through Investigation two.

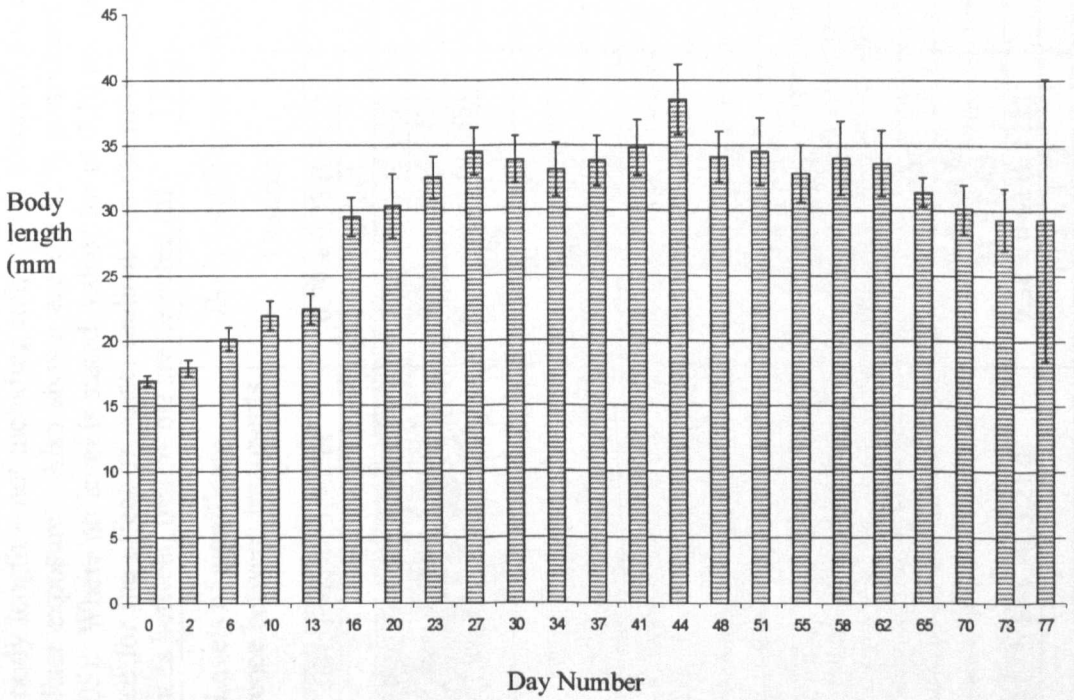


Table 6.8. Mean body length – not including tail; (mm) recorded for developing larvae from stage 22 through to stage 42 (Gosner;1960) for each level of fertiliser exposure. Also shown are the 95% confidence intervals and the results of analysis of covariance with the levels of significance ($p<0.05$). Where no level is stated, values for $p>0.10$ and no significant differences between treatments were detected. The p value level of significance for the effect of vessel replicate on the mean body length (mm) is shown in brackets alongside each mean value where significant differences between treatments are detected (df = 7, 1343).

Day number	[$]Levels of significant difference between treatments	Body length (mm) with 95% confidence intervals for each nitrate (NO_3^--N) exposure concentration.			
		Control	25mg/l	50mg/l	100mg/l
0	p=<0.001; F=8.90; 3,92 [£]df***	6.79 ± 0.33 (0.333)	7 ± 0.28 (0.390)	5.96 ± 0.42 (0.993)	6.88 ± 0.26 (0.053
2	-	7 ± 0.41	6.5 ± 0.46	6.67 ± 0.35	6.38 ± 0.24
6	p=0.004; F=4.66; 3,92 df***	7.83 ± 0.32 (0.943)	8.04 ± 0.55 (0.602)	7.25 ± 0.36 (0.493)	7.21 ± 0.33 (0.674)
10	p=<0.001; F=7.29; 3,92 df***	9.21 ± 0.35 (0.645)	9.13 ± 0.63 (0.969	8.25 ± 0.38 (0.596)	8 ± 0.46 (0.491)
13	p=<0.001; F=8.70; 3,92 df***	9.13 ± 0.61 (0.240)	9.38 ± 0.67 (0.787)	9.04 ± 0.38 (0.493)	7.67 ± 0.44 (0.257)
16	-	9.29 ± 0.52	9.4 ± 0.47	9.46 ± 0.37	9.33 ± 0.24
20	-	10.5 ± 0.69	11 ± 0.48	10.7 ± 0.9	10.4 ± 0.89
23	-	10.4 ± 0.53	10.8 ± 0.55	9.96 ± 0.49	10.3 ± 0.64
27	-	10 ± 0.38	10.4 ± 0.49	9.96 ± 0.26	10.4 ± 0.39
30	-	10.4 ± 0.22	10.3 ± 0.46	10.4 ± 0.36	10.6 ± 0.50
34	-	10.4 ± 0.38	10.8 ± 0.44	10.6 ± 0.39	10.3 ± 0.46
37	-	10.6 ± 0.38	11.3 ± 0.72	11.1 ± 0.45	11.3 ± 0.53
41	-	11.1 ± 0.94	11 ± 0.89	11 ± 0.78	11.5 ± 1.67
44	-	10.4 ± 0.43	11.1± 0.83	11.1 ± 0.94	11.1 ± 1.14
48	-	10.1 ± 0.94	10.5 ± 0.64	10.6 ± 0.89	10.1 ± 0.83
51	-	10.4 ± 0.77	11 ± 1.00	10.5 ± 0.78	11.3 ± 0.74
55	-	10.3 ± 0.70	10.6 ± 1.18	10.4 ± 1.00	10.9 ± 1.14
58	-	11.7 ± 0.86	12.1 ± 0.94	12 ± 1.63	12.3 ± 0.86
62	-	11.4 ± 0.63	11.6 ± 1.05	12.3 ± 0.45	12 ± 1.10
65	-	31 ± 2.48	33.2 ± 3.00	33 ± 3.39	31.8 ± 2.04
70	p=0.013; F=5.70; 3,21 df***	9.38 ± 0.62 (0.112)	9.38 ± 1.27 (0.356)	11.3 ± 3.80 (0.632)	10 ± 0.55 (0.079)
73	-	10.3 ± 1.53	10.8 ± 1.04		10 ± 1.30
77	-	9.67 ± 1.44	10.3 ± 0.80		10.3 ± 1.24

[$] One-way analysis of variance: p values ***<0.05 considered significant. Values *>0.05 &<0.1 were considered to reflect trends .

[£] df = degrees of freedom (n-1).

Figure 6.13. Mean body length – not including tail (mm) for Control (untreated) larvae with 95% confidence intervals, for flow-through Investigation Two.

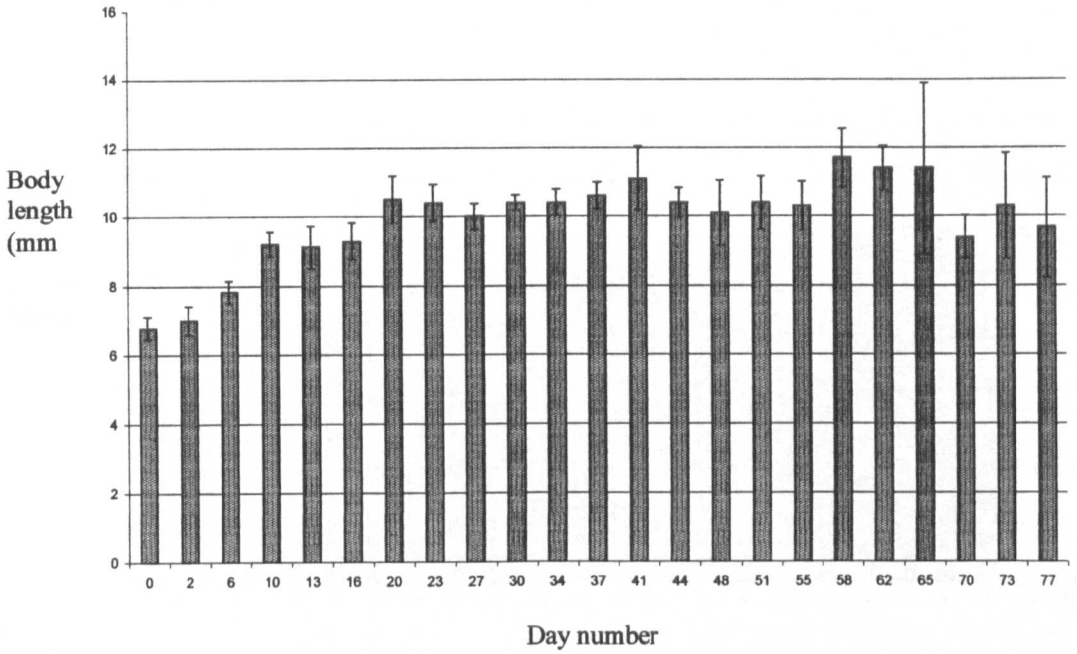


Figure 6.14. Mean body length – not including tail (mm) for 25mg/l NO_3-N treated larvae with 95% confidence intervals, for flow-through Investigation Two.

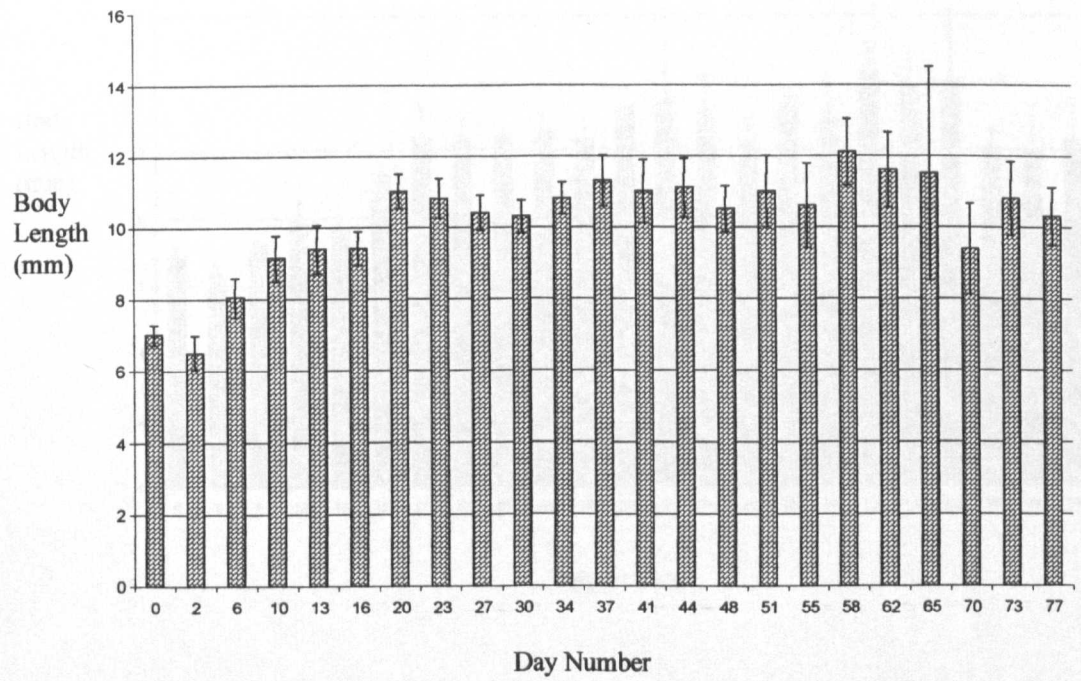


Figure 6.15. Mean body length – not including tail (mm) for 50mg/l NO_3-N treated larvae with 95% confidence intervals, for flow-through Investigation Two.

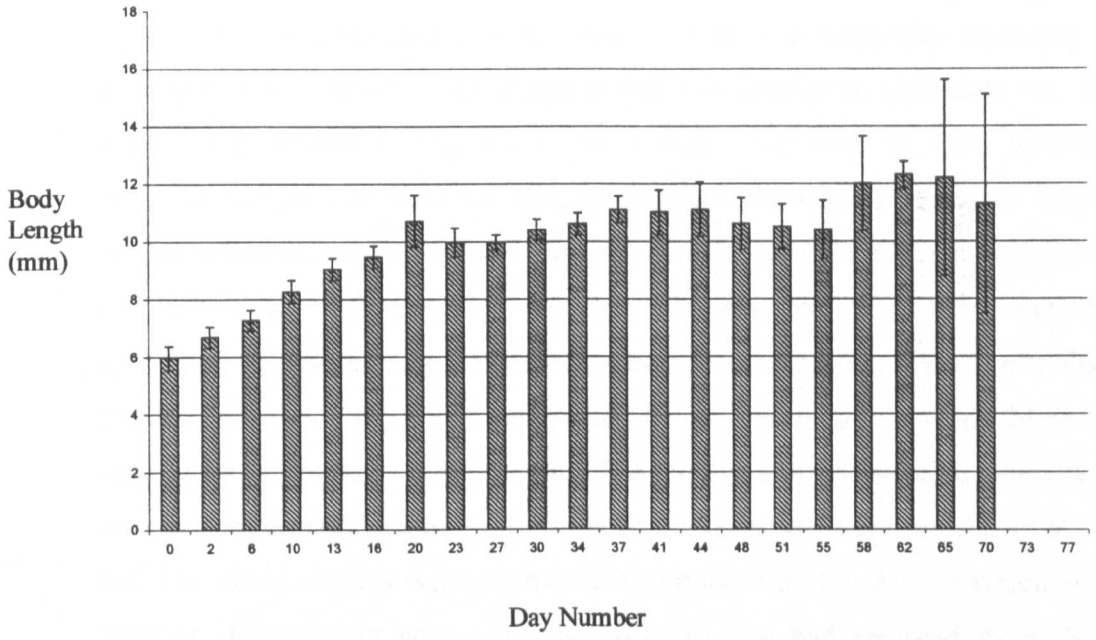
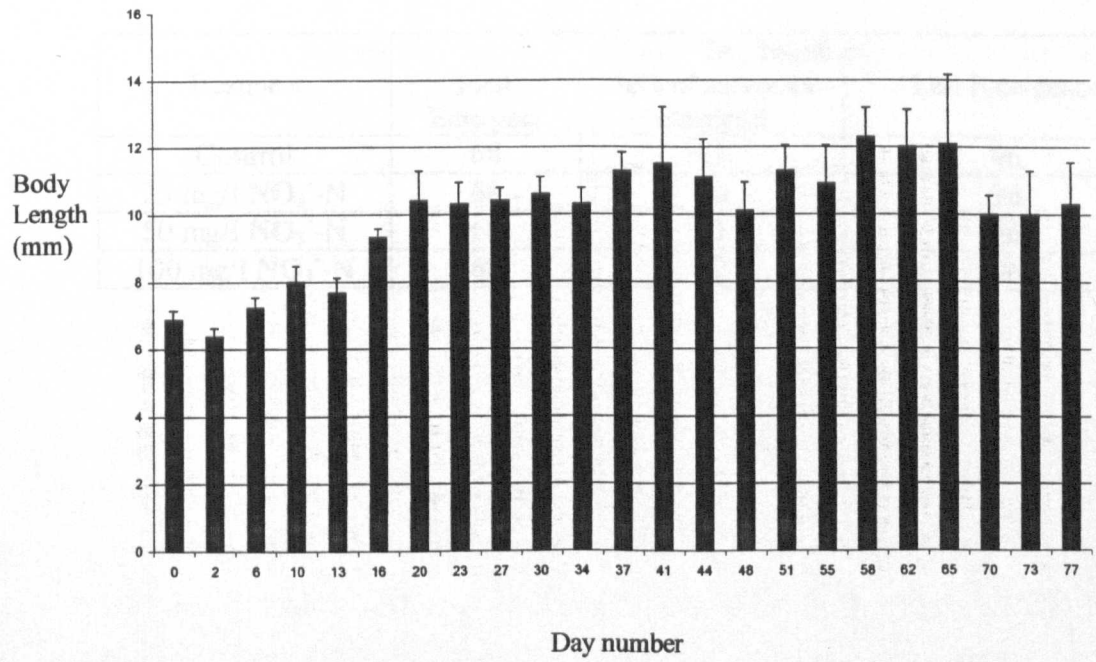


Figure 6.16. Mean body length – not including tail (mm) for 100mg/l NO_3-N treated larvae with 95% confidence intervals, for flow-through Investigation Two.



6.5.4 Metamorphic emergence rates and body mass at metamorphosis

The daily emergence rates and cumulative emergence rates are presented in Table 6.9. The daily number of metamorphs that emerged from each test concentration were plotted as a percentage of the total number of metamorphs emerging from each test concentration. Linear regression was applied to each data set. These plots are presented in Figures 6.17a – 6.21. The time to 50% metamorph emergence in days at each test concentration are shown in Table 6.10. The daily emergence rates were compared using Students't'-tests for between treatments to establish if any differences in the rates of emergence. The larval period for individuals exposed at each exposure concentration was established. Metamorphic body mass was determined for each metamorph within 24 hours of emergence from the test system. The daily and cumulative numbers of metamorphs emerging from each of the four treatment groups are shown in Table 6.9. The larval periods were estimated by establishing the day on which at least 50% of all surviving larvae at each concentration had emerged from the test system.

Table 6.10 Larval periods for larvae at each ammonium nitrate exposure concentration. The time for complete metamorphosis of all surviving larvae is also shown.

Treatment	Day Number		
	First Emergent	50% of survivors emergent	Last Emergence
Control	68	81	96
25 mg/l NO_3^--N	68	80	96
50 mg/l NO_3^--N	68	81	96
100 mg/l NO_3^--N	68	82	96

Table 6.9 Daily emergence rates for metamorphs at each ammonium nitrate exposure concentration. Values in bold text are approximate 50% emergence values.

Day	**Met	Exposure Concentration (NO_3^--N)											
		Control			25 mg/l			50 mg/l			100 mg/l		
N°	Day	*N°	$C	#%	N°	C	%	N°	C	%	N°	C	%
68	1	11	11	17	11	11	18	3	3	5	2	2	5
71	4	3	14	21	1	12	20	4	7	13	2	4	11
78	11	7	21	32	8	20	33	5	12	21	3	7	18
79	12	4	25	38	7	27	44	7	19	34	1	8	21
80	13	0	25	38	2	29	**48**	0	19	34	1	9	24
81	14	9	34	**52**	8	37	61	12	31	**55**	7	16	42
82	15	7	41	62	7	44	72	5	36	64	3	19	**50**
83	16	2	43	65	0	44	72	0	36	64	0	19	50
84	17	3	46	70	2	46	75	4	40	71	4	23	61
85	18	2	48	73	1	47	77	1	41	73	2	25	66
88	21	5	53	80	4	51	84	9	50	89	8	33	87
89	22	1	54	82	1	52	85	2	52	93	0	33	87
90	23	0	54	82	7	59	97	2	54	96	2	35	97
93	26	7	61	92	2	61	100	2	56	100	3	38	100
96	29	5	66	100	-	-	-	-	-	-	-	-	-
Total N° of metamorphs		66			61			56			38		

*N = number of metamorphs emerging on each day
$C = cumulative number of emerging metamorphs
#% = cumulative number of emerging metamorphs expressed as a % of the total number of metamorphs emerging from each treatment.
**Met day = metamorph emergence day after the presence of the first metamorphs.

No significant differences were established between treatments relative to the controls ($p>0.05$, $df = 29, 28$ & 28 for control vs 25, 50 and 100 mg/L treatments respectively). From Table 6.10, no significant differences were detected between the larval periods based on the surviving larvae from each of the treatment groups. Figure 6.17 displays regression lines that reveal the % daily rate of metamorph emergence being slightly reduced at 50 and 100 mg/L NO_3^-N when compared to the control treatment. All surviving larvae emerged from the test system within 2 days of each other. Metamorphic emergence rates were compared by regression analysis, whereby the cumulative number of emergent metamorphs, were plotted against time. Cumulative emergence rates in the control, 25 and 50mg/l treatment groups are similar, with a reduced rate shown at the high dose (100mg/l), were established and are presented in Figure 6.21.

Figure 6.17 Daily metamorph emergence (%) over the duration of the flow through Investigation Two, revealing reduced rates of emergence with increasing concentration of ammonium nitrate.

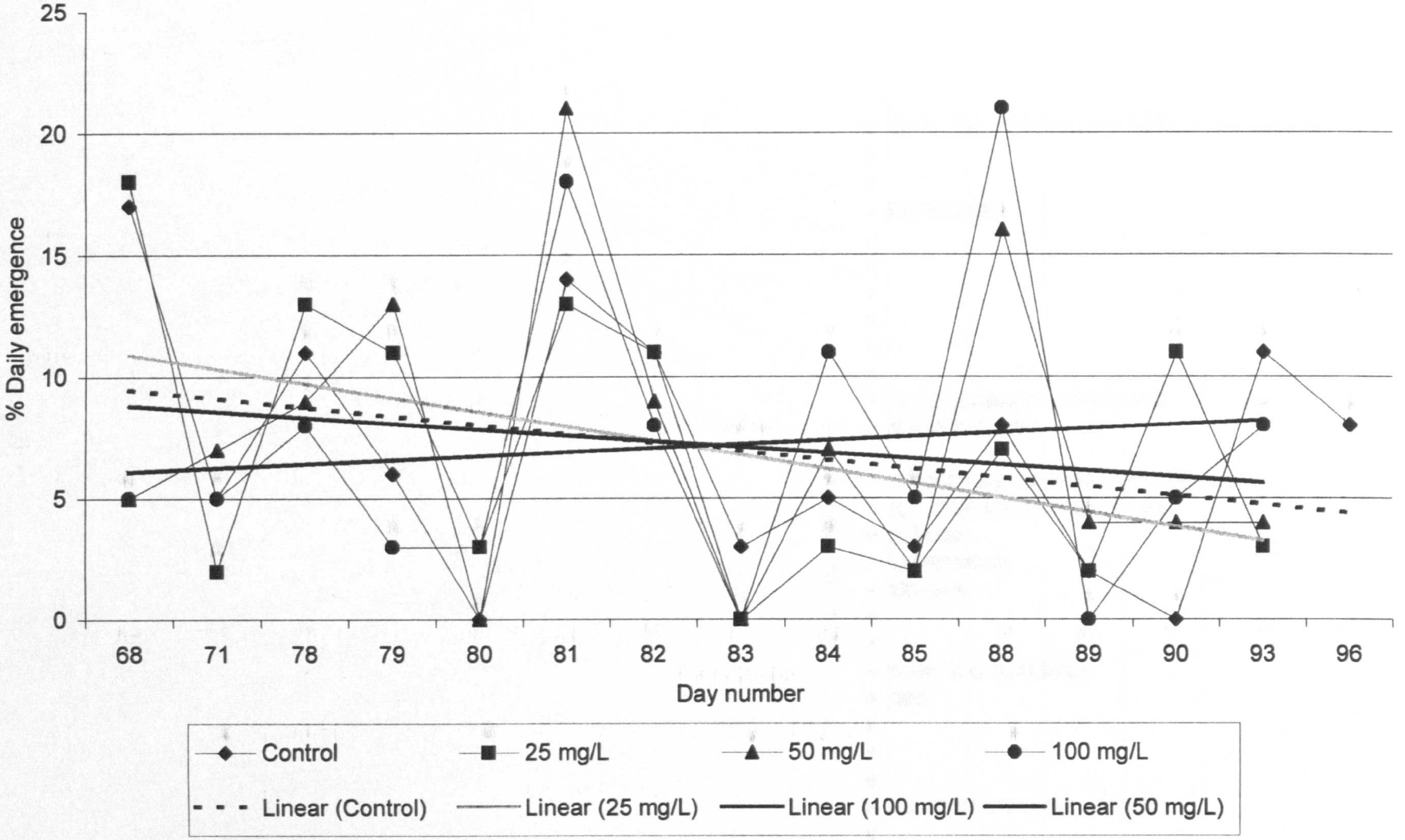


Figure 6.17a Numbers of emergent metamorphs and the days of emergence for frog larvae exposed under control conditions.

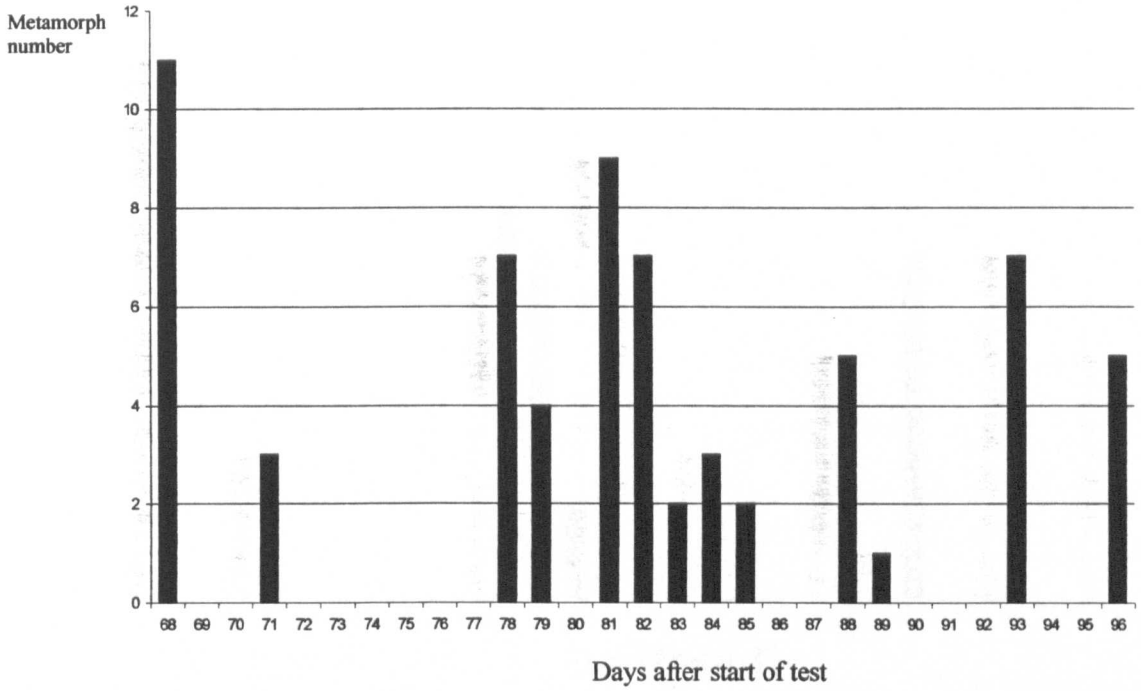


Figure 6.18 Numbers of emergent metamorphs and the days of emergence for frog larvae exposed at 25mg/l NO_3-N.

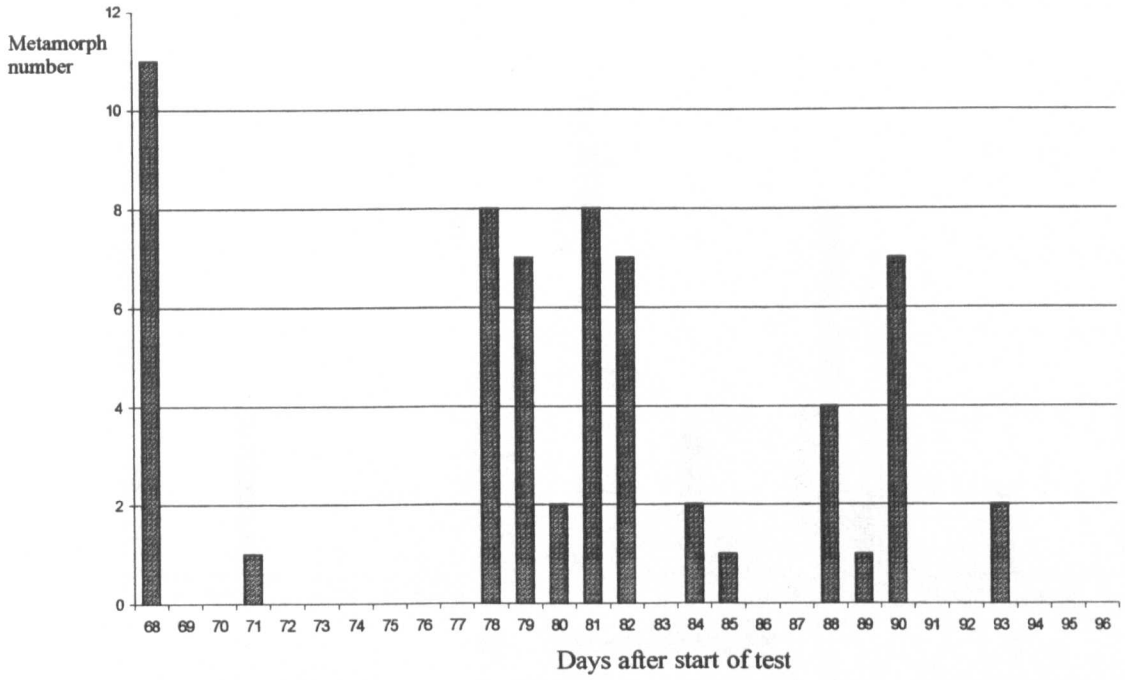


Figure 6.19 Numbers of emergent metamorphs and the days of emergence for frog larvae exposed at 50mg/l NO_3-N.

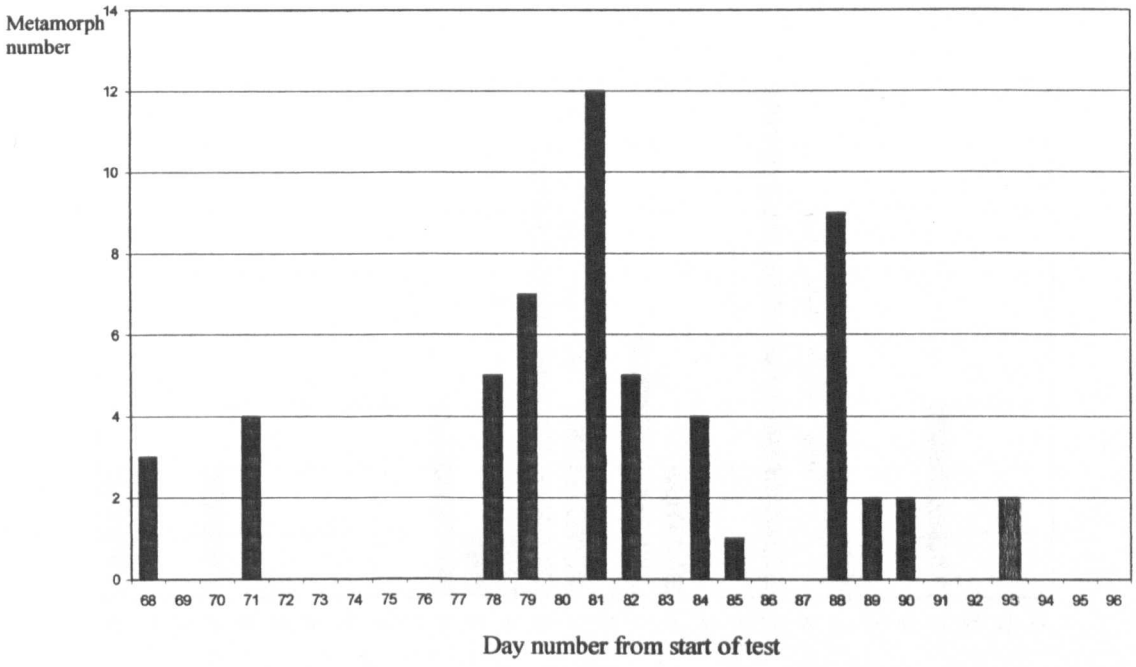


Figure 6.20 Numbers of emergent metamorphs and the days of emergence for frog larvae exposed at 100mg/l NO_3-N

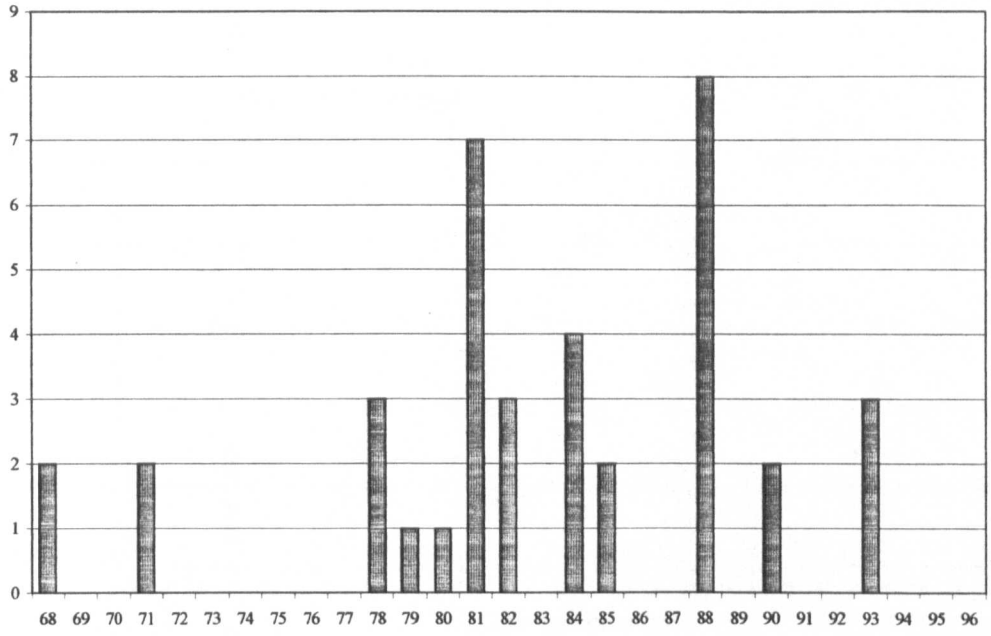
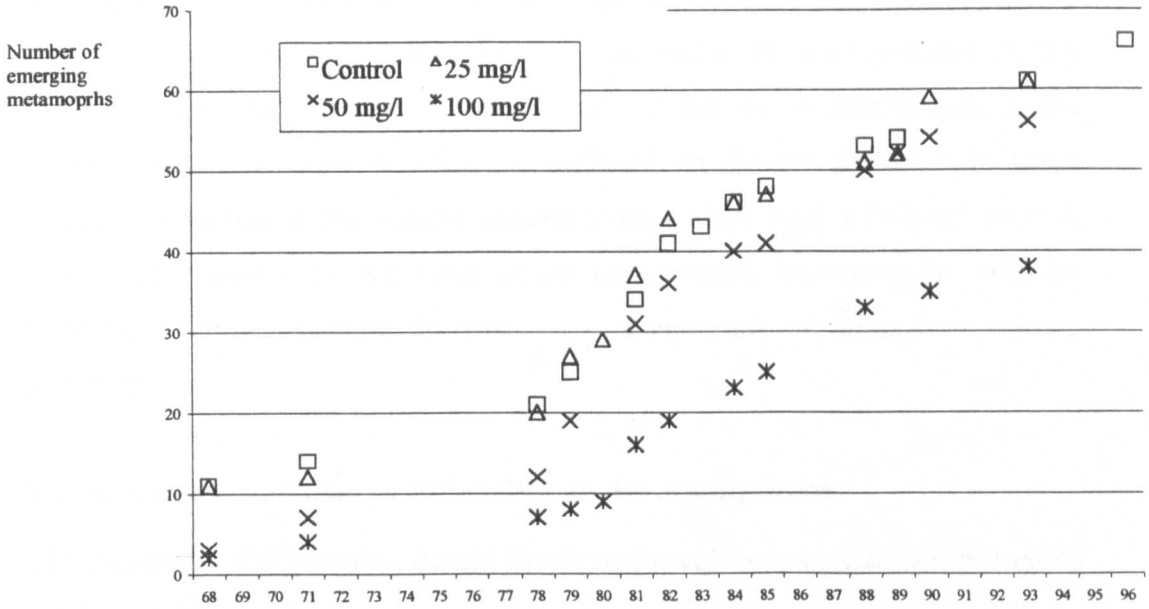


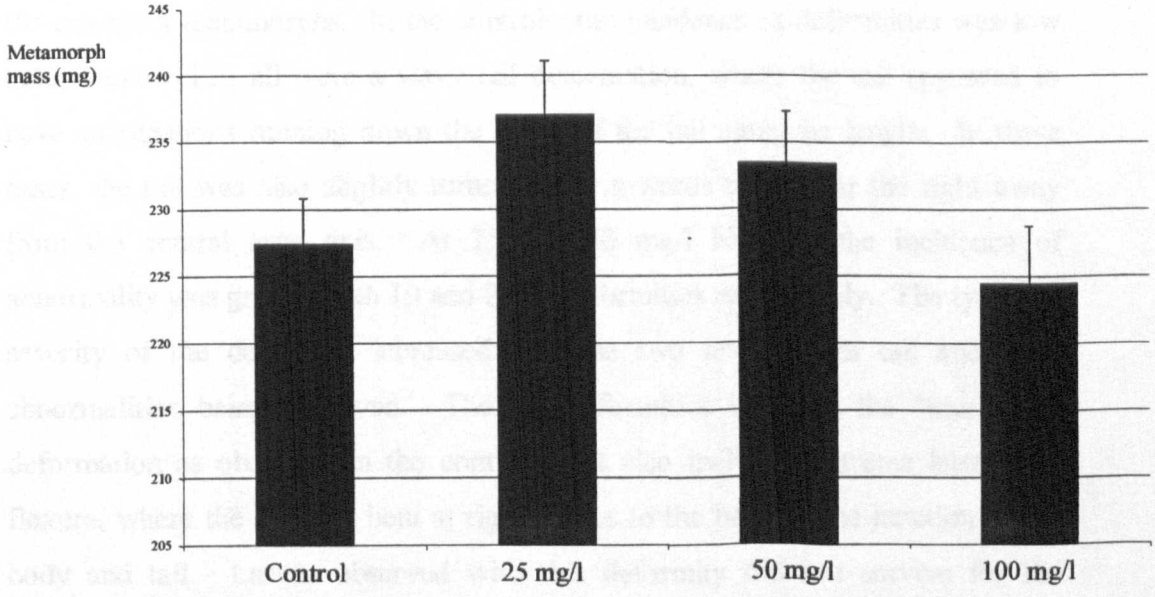
Figure 6.21 Cumulative metamorph emergence rates with correlation coefficients for each treatment group.



Regression Correlation coefficients

Treatment	Control	25 mg/l NO_3-N	50 mg/l NO_3-N	100 mg/l NO_3-N
Correlation values (r)	0.952	0.939	0.943	0.893

Figure 6.22 Metamorph mass (mg) at emergence from the test system at each exposure concentration (95% confidence intervals shown).



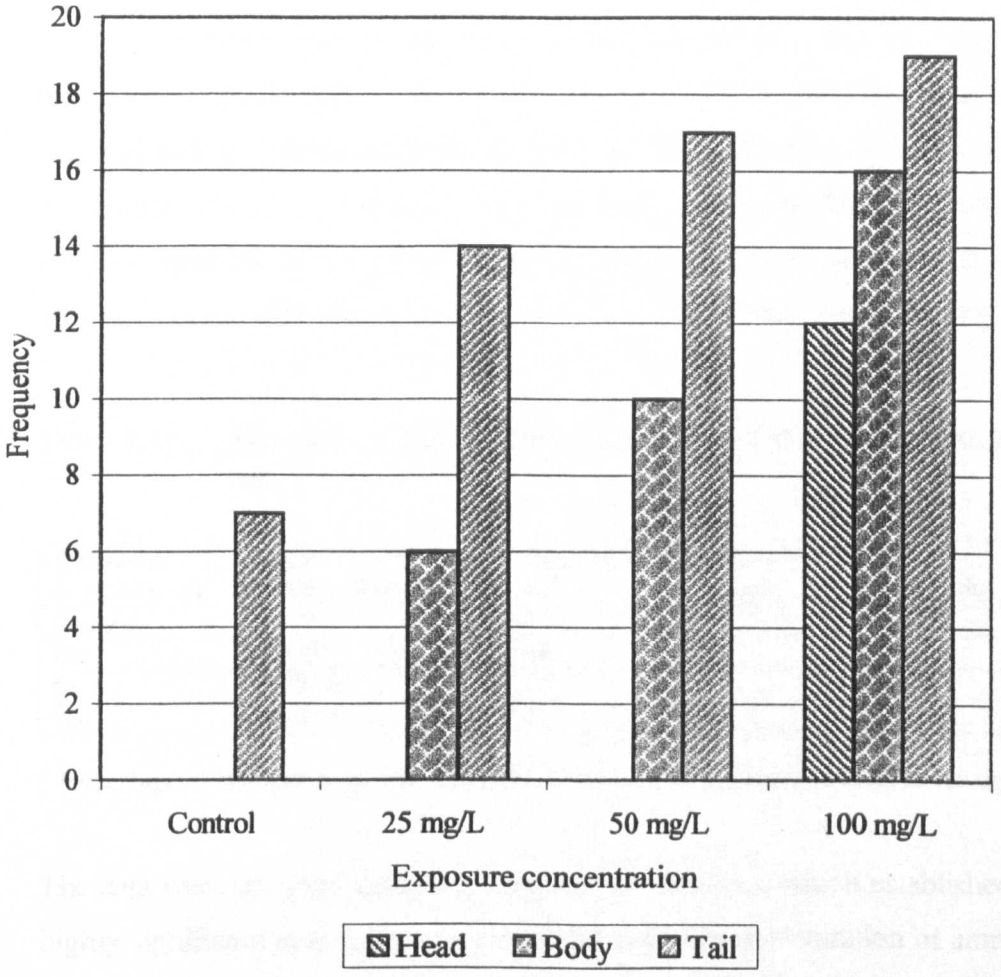
Body masses at metamorphosis were measured within 24 hours of emerging from the test system. The means are displayed in Figure 6.22. The results of an analysis of variance test reveals significant differences in the body mass between control and emergent metamorphs across concentrations (F=0.47, p=0.046 (3, 206 df). A Tukey analysis further reveals that the masses of metamorphs in the 25 mg/L treatment were significantly different (at the p=0.05 level) to those metamorph masses at the highest concentration of 100 mg/L (T=10.09, df = 4, 206). From figure 6.21, the trend across the exposure concentrations was for metamorph body mass to decrease as concentration of ammonium nitrate increased.

6.5.5 The type and occurrence of deformities in developing larvae

The frequency of deformities during the flow-through investigation are presented in Figure 6.23.

During the flow through test, inspections of larvae in each of the test vessels reveal that some larvae were deformed. The deformities were broadly grouped into either head, tail, or body deformations. Where larvae survived to metamorphosis with either a head or body deformity, deformities were evident in the emergent metamorphs. In the controls, the incidence of deformities was low (9%, Table 6.12), all were a wavy tail deformation, where the tail appeared to have corrugations running down the depth of the tail along its length. In these cases, the tail was also slightly turned either towards the left or the right away from the central long axis. At 25 and 50 mg/l NO_3^--N, the incidence of abnormality was greater with 19 and 28 % deformities respectively. The type and severity of the deformity increased at these two levels, with tail and head abnormalities being observed. The tail deformities included the 'wavy' tail deformation as observed in the controls, but also included extreme lateral tail flexure, where the tail was bent at right angles to the body at the junction of the body and tail. Larvae observed with this deformity did not survive for the duration of the test. Where head deformities were observed they were

Figure 6.23 Total number of larval deformities defined by type during flow through Investigation Two, for each exposure concentration.



deformities of the mouth and eye. The ocular deformities included: no eye present, a single eye present, or where the eye had developed, there was a layer of skin over the eye preventing proper function. In one larva, there were lesions and raised areas on the skin on the top of the head. Mouth deformities involved poor development of the mouthparts with the opening being much smaller than for normal developed larvae. At the 100 mg/l NO_3^--N highest concentration, deformities were observed in 43% of larvae, with tail deformity being the most common. Head and body deformities were present. Many affected larvae exhibited all three types of deformity. Head deformities were as described for the lower concentrations. Where deformities of the body were present these involved lesions and lumps along the body surface. Others included openings in the ventral side of the gut cavity. In one case, the rudimentary gut was clearly visible outside the body cavity (a hernia), with the animal still alive and actively feeding. Larvae with this deformity did not survive the duration of the test.

Table 6.11 Summary of larval deformities observed during the flow through test.

Treatment (mg/l NO_3-N)	Total N° of deformed larvae	Type and frequency of deformity		
		Tail	Head	Body
Control	7(9)*	7	-	-
25	15(19)*	14	-	6
50	22(28)*	17	-	10
100	34(43)*	19	8	16

* Percentage ( ) of deformed larvae at each treatment level as a percentage of all larvae exposed

The data were analysed using a chi-squared (χ^2) analysis, which established that a highly significant association does exist between the concentration of ammonium nitrate and the frequency of abnormalities observed in the test vessels (see Table 6.12) during the test; $\chi 26 = 31.70$; df = 6; p = 0.05. Closer inspection of the contingency table reveals that for each type of abnormality, where they occur, the highest χ^2 values reveal which type of abnormality are most prevalent at each of the exposure concentrations.

Table 6.12 Contingency table for abnormality frequency during flow-through test.

Treatment (mg/l NO_3-N)	Parameter	Type and frequency of deformity			Observed Totals
		Tail	Head	Body	
Control	O	7	0	0	7
	E	4.11	0.577	2.31	
	χ^2	2.03	0.577	2.31	
25	O	14	0	6	20
	E	11.8	16.5	6.60	
	χ^2	0.410	16.5	0.065	
50	O	17	0	10	27
	E	15.9	2.23	8.91	
	χ^2	0.076	2.23	0.133	
100	O	19	8	16	43
	E	25.3	3.55	14.2	
	χ^2	1.57	5.58	0.228	
	Observed Totals	57	8	32	97

In the control treatment, by default, tail abnormalities are the most abundant. In the 25 mg/L, the highest χ^2 value is associated with tail abnormalities ($\chi^2 = 0.410$). In the 50 mg/L treatment, the highest χ^2 is associated with body abnormalities ($\chi^2 = 0.133$). In the 100 mg/L treatment, the highest χ^2 value is associated with head abnormalities. Where abnormalities occur, the highest χ^2 (7.38) is associated with the 100 mg/L treatment, concluding that there is an association between the higher concentrations of ammonium nitrate and the frequency of abnormalities observed in developing larvae. Of the abnormality types, most prevalent in this study are those abnormalities associated with the tail

6.6 DISCUSSION

The use of a flow through or dynamic apparatus was successfully used to study of frog larvae over prolonged periods in the laboratory. The use of the testing system evolved during the course of the research, establishing that mortality rates were dependent on high maintenance of the test system. However, it was determined from a shortened duration of the first flow-through test, concentration of NO_3^--N was seen to have a significant impact on larval body mass, total body length and body length.

Following a more efficient test system maintenance regime, the system was more robust. Increasing fertiliser concentrations were found to have a significant impact on larval survival. The most sensitive phase in tadpole development in terms of survival was between front limb emergence and final metamorphosis, where the highest mortality rates were recorded. Increasing fertiliser concentration during the exponential growth phase was found to have a significant effect on body mass, body and total body lengths.

Measured concentrations of residual NO_3^--N were adequately achieved between 80 and 120% of the nominal concentrations within the test system. The majority of surviving larvae emerged as metamorphs between days 77 and 89 of the test. No significant differences were found between the rates of metamorphic emergence (larvae at 100mg/l NO_3^--N showed a slightly reduced rate of emergence), the time of first emergence, the time at which 50% of the surviving larvae emerged and the time of final larvae emerged from the test system. No significant differences were detected in the mass of metamorphs emerging from the test system, although, metamorphs at 100mg/l NO_3^--N were significantly smaller than those emergent metamorphs at 25 mg/l NO_3^--N; these were not significantly different from the controls. The number and severity of deformities increased with fertiliser concentration.

CHAPTER SEVEN

THE EFFECT OF AMMONIUM NITRATE FERTILISER ON LOCOMOTORY ACTIVITY AND AVOIDANCE BEHAVIOUR IN ADULT COMMON FROGS (*Rana temporaria*)

CHAPTER SEVEN

THE EFFECT OF AMMONIUM NITRATE FERTILISER ON LOCOMOTORY ACTIVITY AND AVOIDANCE BEHAVIOUR IN ADULT COMMON FROGS '*Rana temporaria*'

7.1 INTRODUCTION

When a frog jumps, it maximises the distance travelled in a unit of time. This may be to avoid the interests of a predator, to avoid a source of stress, or move between suitable habitats in search of a refuge or food. Jumping in common frogs may be defined as a voluntary movement where all four limbs are airborne. The relationship between anuran morphology and jumping distances is now better understood, by the correlation of body mass, body lengths and limb lengths in a range of anuran species. Emmerson (1978) related forms of anuran locomotion with particular morphology in many anuran species. Amphibians with low hind limb to snout vent ratios, tended towards walking as their dominant mode of locomotion, whereas species with high ratios had jumping as their dominant mode of locomotion. All species tested appeared to increase their jump distance with increasing body size. Variations in the relationship between body size and jumping distance may be used as an indicator of a change in individual behaviour, following changes in environmental conditions, or in individual physiological response, following perturbation such as may be encountered in a pollution episode. Modified locomotory behaviour may increase the risk of predation by making individuals more susceptible, reduce an individuals chance of successfully reproducing by preventing individuals reaching suitable breeding sites, and so reducing its reproductive fitness.

For wild populations, where body mass and stature are positively correlated, it may be possible to detect pollutant affects by monitoring individual locomotory ability, or predict the possible effects of exposure to a pollutant such as

ammonium nitrate, by observing adult frogs in the laboratory under controlled conditions. Changes in a frog's ability to move, or a significant change in jumping distances may therefore be used as an indicator of stress.

Behavioural changes such as reduced swimming ability, involuntary body twitching, loss of balance or changes in feeding rates in amphibian larvae have been reported as effects, following exposures to pesticides. These include the following compounds: carbofuran (Takeno *et al.*, 1977); and malathion (Kowsalya *et al.*.1987). These chemicals are neurotoxic in their mode of action, affecting the central nervous system and peripheral nervous system. This type of behavioural change has the potential to increase the chances of predation, as has been shown with the crested newt predation on frog tadpoles (Cooke 1970). An individual adult may well be able to overcome the direct affects of the pollutant and successfully reproduce within that habitat, but the effect may well manifest in an individual's progeny. This may present as reduced larval survival due to direct affects on the integrity of the spawn jelly sacs, or the processes involved in larval development may well be impaired following exposure to these pollutants reducing subsequent survival.

Investigations into the locomotory activity and avoidance behaviour of adult common frogs were carried out in the laboratory. The aim was to test the following hypotheses:

> 'A positive correlation exists between the level of frog activity and the concentration of ammonium nitrate fertiliser to which an individual frog has been exposed.'

> 'Avoidance behaviour in adult frogs can be modified by exposure to ammonium nitrate fertiliser.'

7.2. METHODS

7.2.1 Avoidance and activity test chamber

Activity levels and fertiliser avoidance were investigated simultaneously using an avoidance / activity chamber (Figure 7.1). For each test, two identical chambers were used. One chamber was used as a control, to which no fertiliser was applied at any time. The second chamber was the exposure chamber, in which fertiliser was applied. Each chamber was a constructed glass aquarium (29cm x 75cm x 25cm) that was divided into two halves by a 1cm high clear-Perspex barrier that was secured using aquarium silicon sealer. This divided the tank equally in half, and prevented the flow of any solution between the two halves of the tank. Into each half was placed a substrate of plain white blotting paper. This was chosen because after wetting would maintain a sufficient level of moisture within the tank. The test chamber was maintained under the same conditions as the frog holding tanks. The test chambers were isolated in a room behind a screen (a large plain-white linen bed sheet). Through a hole in the centre of the sheet, the lens of a closed circuit television camera was inserted. This was connected to a 26-inch television monitor. The use of the camera prevented disturbance to individual frogs during the 2.5-hour testing period.

7.2.2 Activity levels.

Activity levels of individual adult frogs were monitored periodically during the 2.5-hour testing period. The first 30 minutes were used as an acclimation period. The subsequent two hours were split into two distinct monitoring periods. The first hour was used a control hour, where no fertiliser was applied. In the second hour was the exposure period, when individuals were exposed to fertiliser. In each hour following the acclimation period, frog activity was scored at 5-minute intervals. A four-point ordinal scale of activity was used. This was based on observations of captive held adults in the laboratory. The criteria of activity are shown in Table 7.1. Activity scores were analysed for each frog at each exposure concentration, using a non-parametric method (Kruskal-Wallis). Each frog was used once, with the activity score for each frog during the exposed hour of the test

being compared to the control hour. Data were pooled for final analysis and presentation of results.

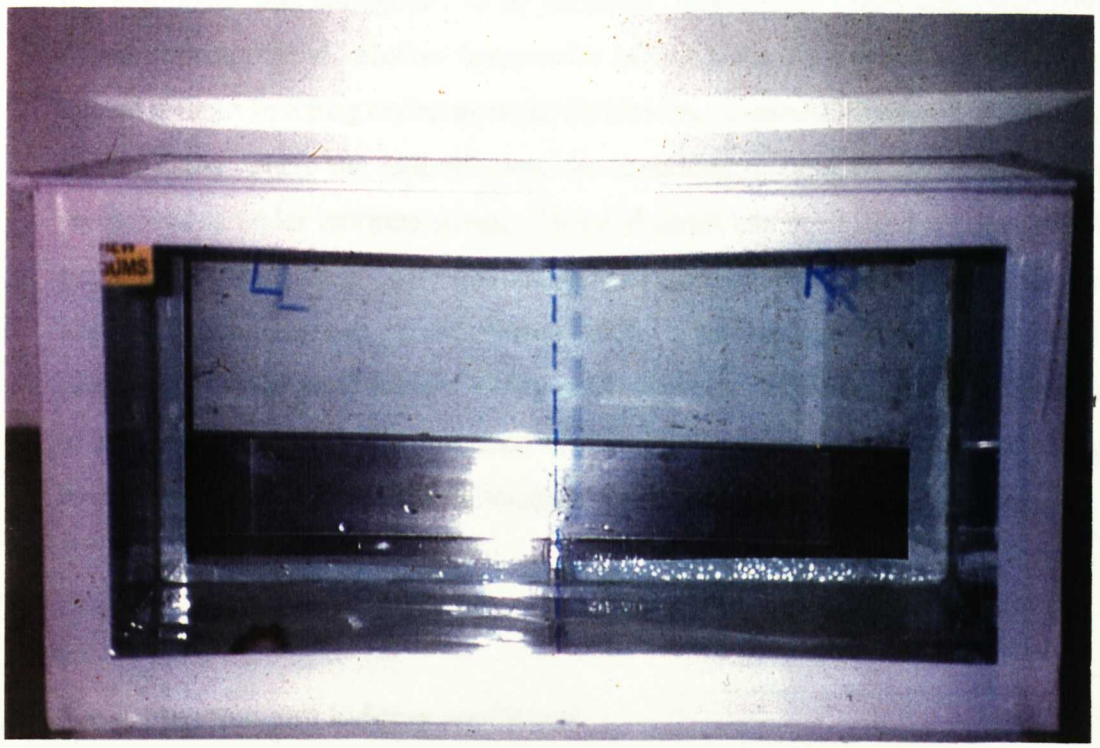
Table 7.1. Ordinal activity scale for scoring individual frog activity (notes on ventilation are also included).

Activity level.	Definition
1.	Frog stationary with the ventilation activity dominated by buccal movements.
2.	Frog in a semi-stationary position; not moving further than a body length in distance, but turning on the spot, with the ventilation activity being dominated by buccal movements.
3.	Frog not stationary, but is actively moving around the tank either by means of walking or short hops < 3 x body length. Lung movement frequency =< buccal movements.
4.	Frog not stationary. Frog is jumping with hops > 3 x body length. Frog has extending hind limbs that do not return to a normal retracted position after extension. Lung ventilatory activity is noticeably altered, with lung movements = buccal movements; flared nares.

7.2.3 Avoidance behaviour

Frog location was identified as either a '1' or a '2'. Hlaves of the test chamber were randomly identified using number tables from left to right to allocate fertiliser to a half within the chamber. At each exposure level, four individual frogs were exposed under test conditions. By monitoring the closed circuit television, the time spent in each half of the test chamber was determined by recording time after start of test, at which the frog moved to the other half of the tank. At the end of the testing period, the total time spent in either half '1' or '2' of the tank was established.

Plate 7.1 Adult Common Frog preference / activity chamber used to assess the effect of ammonium nitrate on individual preference / avoidance behaviour and activity levels.



The use of the closed circuit camera within an isolated testing arena permitted close monitoring of frog activity and location whilst allowing ventilation activity to be monitored to assess levels of stress. Lung and buccal movements were monitored for the duration of the test. After the first hour, the individual was swiftly removed from the tank, and placed into the second tank containing a known exposure level of fertiliser (or control). After a 2-minute acclimation period, scoring of the frog continued for another hour. The test was terminated if the individual was considered to be suffering excessively. This was established by monitoring the ventilation frequencies between the lung-breathing reflex and buccal reflex. In a frog under stressful conditions, research has shown (Oldham *et al.* 1997) that where the ratio of these two breathing techniques approaches unity, the animal is under extreme stress. This end point has been used successfully in preventing the death of animals during exposure to fertilisers. When this was observed, the frog was quickly removed to a bucket and rinsed with freshly prepared artificial pond water (APW) until recovery. The frog was then returned to a holding tank and was not used further in the testing procedure. All frogs were starved for 24 hours before introduction into the test chamber.

7.2.4 Frog collection and holding conditions

Adult frogs were collected from the field (Coleorton, North West Leicestershire) using pitfall traps. Individuals were weighed on collection. They were held in the laboratory for at least a week before exposure to acclimatise to experimental conditions. They were held at 16 ± 2°C with a relative humidity > 50%, in 10-litre plastic aquarium on a moist substrate of wetted blotting paper with refuge (plastic plant pots). They were fed using domestic house crickets (*Acheta domestica)* at a feeding rate of 2% of individual frog body mass per day.

7.2.5 Start of test procedure

Each frog was removed from the holding tank, placed into a bucket of artificial pond water, and transferred to the testing room. Frogs were introduced (first to the control chamber) at a central point along the central bar that spanned the width of the chamber. A lid was placed on the chamber and the acclimation period was started. During this period, the frog was monitored continuously for signs of stress as previously described. Once the control hour was complete, the frog was quickly transferred to the exposure chamber where it was again introduced at a central point along the central perspex bar. Test scoring during the second hour was started immediately after the frog was introduced to the chamber.

Each test used two identical testing chambers. All experiments were carried out in a testing facility maintained at a constant temperature for the duration of approximately 16±2° C. The lighting regime was 14 hours light and 10 hours dark lighting regime, with a light intensity of between 500 and 1000 lux. Humidity in the facility was between 55 and 70 %. After the acclimation period, individual frogs were removed from their holding tanks and weighed on a top pan balance in an opaque white bucket (complete with lid). The frog was then placed into the testing arena (Plate 7.1). For each test, the blotting paper was saturated with 55ml of artificial pond water (APW), with water visible on the surface of the blotting paper as small patches. The second tank was prepared in an identical manner, with a known level of fertiliser granules applied to the wetted surface of one side of the tank.

Fertiliser was applied at four exposure concentrations and there was a control. The levels were determined from the information received on the average application rates of ammonium nitrate fertiliser for the Leicester area (Draper *pers comm*). A field application rate of 49 g/sq.m as granules of fertiliser (equivalent to approximately 38 g/sq.m as nitrate) was used as an intermediate level, equivalent to 100% exposure. Nominal exposure levels were set at 50, 100, 150 and 200% (equivalent to 24.5, 49, 73.5 and 98 g/sq.m as granules) of the

intermediate value. These were allocated at random to each test after the random selection of the frog for use in the test.

7.2.6 Avoidance behaviour under field conditions

In an attempt to extrapolate observed affects in the laboratory to a field scenario, avoidance behaviour was investigated in the field using a small number of adult common frogs to demonstrate that frog movements could be modified in the field. To this end, a crop of Winter wheat was sown in October 1995 in a 1.5m^2 enclosure constructed of chicken wire 75cm high covered in a double thickness of polythene sheeting. Approximately 10-15 cm of the fence was buried into the ground. Within the plots, each enclosure was divided into two equal halves using bamboo canes. Mesh netting was placed over the top of the enclosure to exclude birds. Ammonium nitrate fertiliser was applied in granular form, at an application rate equivalent to the recommended field application rate used in the laboratory studies (48 g/sqm).

Fertiliser was applied to one side of the first enclosure in August 1996, at a point when the crop was between 35 and 60cm in height. After application, the crop was watered to aid the initial dissolution of the fertiliser granules (the weather had been consistently warm and dry and soil moisture levels were low). Soil nitrate concentrations were determined before and after fertiliser application. Levels before application (residual) were <25 ppm. Directly after the application of fertiliser at a rate equivalent to 48 g/sqm (54 g per 1.125 sq.m), levels of nitrate in the soil were >100ppm <150ppm.

Three adult frogs (between 20 and 40g mean wet mass) were selected from a common laboratory stock. The mass of each frog was determined and identifying features on the back of each were recorded for ease of identification during the test. All three animals were released into the first enclosure, at a central location towards the middle of the enclosures.

Field trial No. 1

The duration of Field trial 1 was eight days, with fertiliser being applied to the left half of the enclosure in the morning of the 12 August 1996, and then thoroughly watered. Three frogs were then released into the enclosure in the afternoon. Over the next 6 days, the enclosure was visited on four occasions. On each occasion, the wheat was searched extensively, using bamboo canes to gently lay the wheat to one side to help in the search of the individual frogs.

Field Trial 2

In Field trial 2, a finer mesh (1cm) was used to cover the enclosures. The same techniques used for trial 1 were used. The enclosure was visited 12 times over an 18 day holding period. By sampling day 10, one individual frog was missing. To counter this and make the locating of frogs slightly easier, an additional three frogs were added to the enclosure, increasing the density to 5 per 1.5sq.m.
Results for trials No.1 & 2 are shown in Tables 7.4 and 7.5.

It is accepted that statistically, this experiment is not ideal with limited replication of experimental plots because of few individual frogs being available at the time of each of the trials. It does however attempt to demonstrate the potential modification of frog movements in the field following the application of ammonium nitrate under field conditions.

7.3 RESULTS

7.3.1 Activity behaviour.

Frequencies of each activity level during the control and exposure periods are shown in Table 7.2.

Before the first observations were made, each individual frog was allowed 5 minutes to acclimatise at the start of the first hour of the trial. All frogs were maintained under similar conditions to those experienced in the control for a period of not less than 24 hours before the start of the trials.

Table 7.2 Frog activity levels during exposure to 5 concentrations of ammonium nitrate. (n=4 per treatment) (1 = stationary, 2 = semi-stationary, 3 = actively moving & 4 = jumping; refer to Table 7.1 for further detail).

Test Period	Nominal conc. of ammonium nitrate (mg/l as nitrate)	Mean frequency of each activity level.			
		1	2	3	4
1st Hour	0	4	6	0	0
	0	4.5	4.5	0.75	0
	0	5	5	0	0
	0	5.5	4	0.25	0
	0	8	2	0	0
2nd Hour	0	5.5	4.5	0	0
	24.5	3.5	2.5	3.25	0
	49	6	2	2	0
	73.5	4	3	1.75	1
	98	1.25	1	1.25	1.25

The first hour of the trial each of the 4 frogs per test concentration were exposed to the test vessels under 'effectively' control conditions, simulating those conditions experienced in the previous 24 hours of holding within the facility as described. In the first hour of exposure in the test chambers, high frequencies of low activity levels were recorded, with a low frequency of the high activity levels following exposure to fertiliser.

This may be represented using simple histograms with mean frequency of each activity level on the y-axis and the level of activity represented along the x-axis, 1 figure for each hour of the test (Figures 7.1a & b). Figure 7.1a, shows an increased frequency of low levels of activity during the first hour of the test, with almost all frogs displaying low levels of activity within the test chamber. The Figure 7.1b, represents the same frogs, exposed according to fertiliser group allocation. A pattern emerges with control individuals displaying similar levels of activity as were observed in the first hour of the test. For the 50% exposure group, the incidence of low activity levels was reduced, with an increased

frequency of the higher level 3 activity (greater movement around the tank, in smaller hops as opposed to large hops or jumps). For the remaining 2 exposure groups (150 and 200%) there were similar patterns of increased frequency of higher level activity for both groups, although there was low levels of activity still recorded, there was an increase in both activity levels 3 and 4 (hopping and jumping). Mann-Whitney analysis of frequencies of each activity level between the two periods of the test (control and exposed) reveal that for the activity levels 1 and 2 there are no significant differences between the two period across the range of exposure concentrations. However at activity level 3, there is a significant difference between periods of the test (df=5, p=0.0379). At the highest activity level, no level 4 activity was scored during the first period. Between concentrations, a Kruskal-Wallis analysis revealed highly significant differences between activity frequency scores suggesting that the presence of fertiliser led to higher levels of activity across the range of concentrations to which the frogs were exposed over the two periods of testing (H=27.89, df=3, p=<0.01).

From Table 7.2, low activity scores were recorded at the highest exposure concentration. This was due to individual frogs moving into the exposed half of the test chamber and quickly moving back into the unexposed half of the chamber. Here, the frog would remain in a stationary position (activity level 1). In 2 cases, frogs were removed from the test chamber, as the buccal to lung ventilation frequencies were approaching unity. It was noted that the ventral surfaces of these frogs were reddened. At moderate to high concentrations of fertiliser, specific changes in behaviour were also observed. These changes included independent lifting of feet and hind limbs off the substrate.

7.3.2 Avoidance behaviour.

The total time spent in either the control or exposed half of the preference chamber was determined for each fertiliser exposure group. This allowed a

Figure 7.1a. The 1st hour (control period) of the adult activity trials showing combined data for all individuals tested. Ammonium nitrate was not applied during the first hour of the trial.

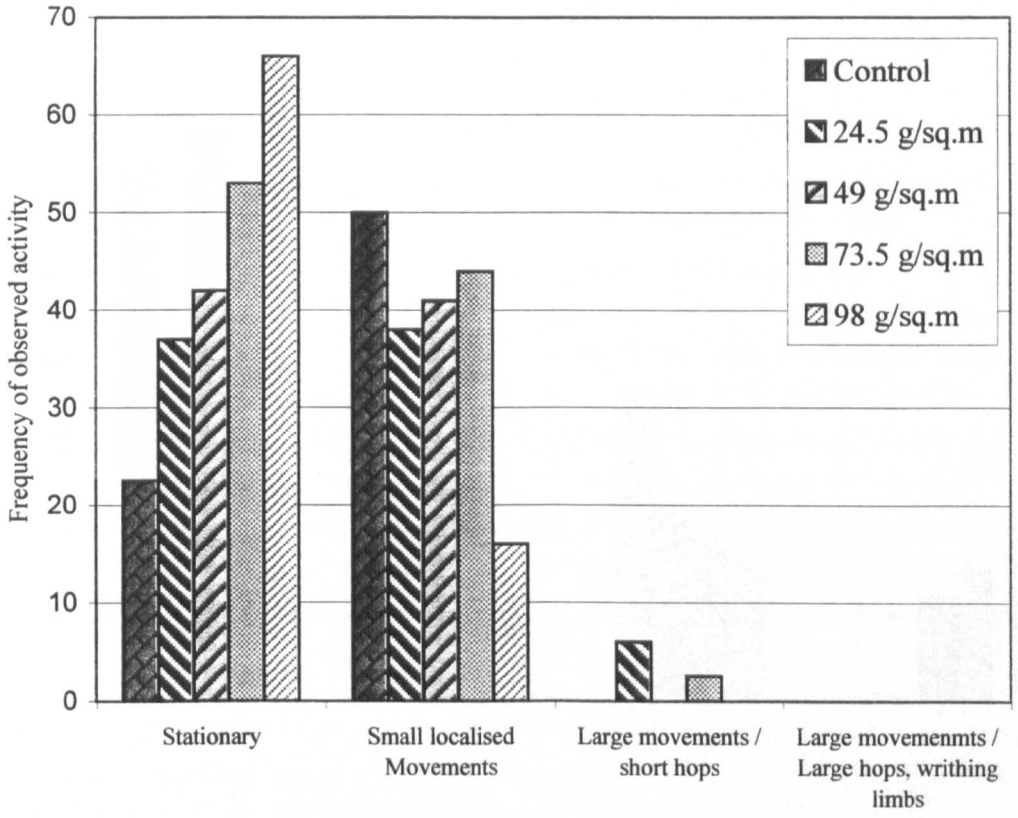
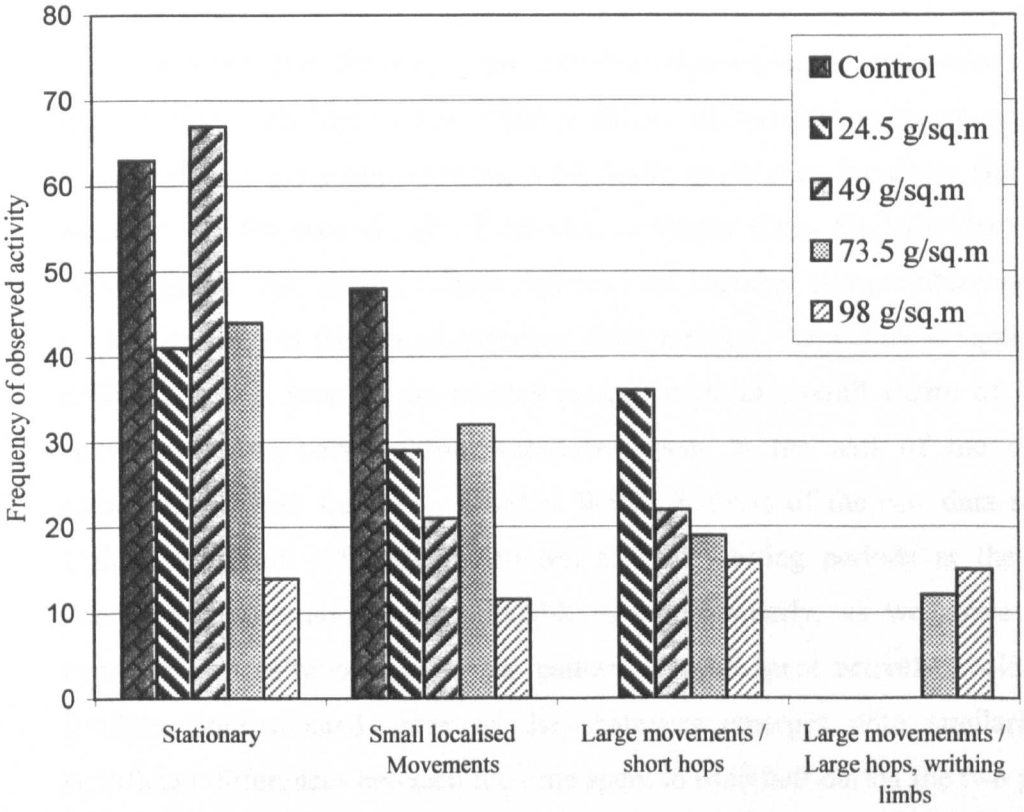


Figure 7.1b. The 2nd hour of the adult activity trials showing combined data for all individuals tested. Ammonium nitrate was applied at rates shown to one half of the exposure chamber.



representation of the data using histograms for each hour of the test. Figure 7.2 displays the proportion of time spent in each half of the chamber. During the first hour (the control period where no fertiliser was applied) and second hour (fertiliser applied according to fertiliser exposure group) of the test, the total time is shown as a proportion of the entire time within the chamber.

From the first half of the test, it may seen that relatively equal amounts of time are spent within each half of the chamber across all fertiliser exposure groups of frogs, with no one group showing a particular preference for either side of the chamber. In the second half of the test, residency times for individuals in the control group were similar in both halves of the chamber as were observed in the control period. At the lowest exposure concentration, the picture is significantly different to that seen in the control period, with an overall figure of 23% of cumulative time between four replicates spent in the half of the chamber contaminated with fertiliser. Kruskal Wallis analysis of the raw data revealed highly significant differences between the two testing periods at the lowest exposure concentration with $p=0.014$, $df=3$. Similarly, as we move up the exposure concentrations, a similar pattern of time spent actively avoiding the fertiliser contaminated areas of the chambers emerges with similarly high significant differences between the time spent in each half during the two periods. Table 7.3 shows the results of the analysis.

The activity and preference experiments were combined to reduce the amount of stress experienced on individual frogs and to reduce the numbers of individual frogs used during the investigations.

Some frogs appeared unable to remove themselves from the contaminated half of the tank. In these cases, the affected frogs were removed from the test chambers to a plastic bucket containing freshly prepared artificial pond. Here they were allowed to recover and then returned to their individual stock tank.

Figure 7.2 Adult location trial displaying the modification of avoidance / preference behaviour with bars representing time spent in each half of the exposure chamber during the control and exposure hour. Ammonium nitrate was applied to the right hand side of the chamber in the 2nd hour only.

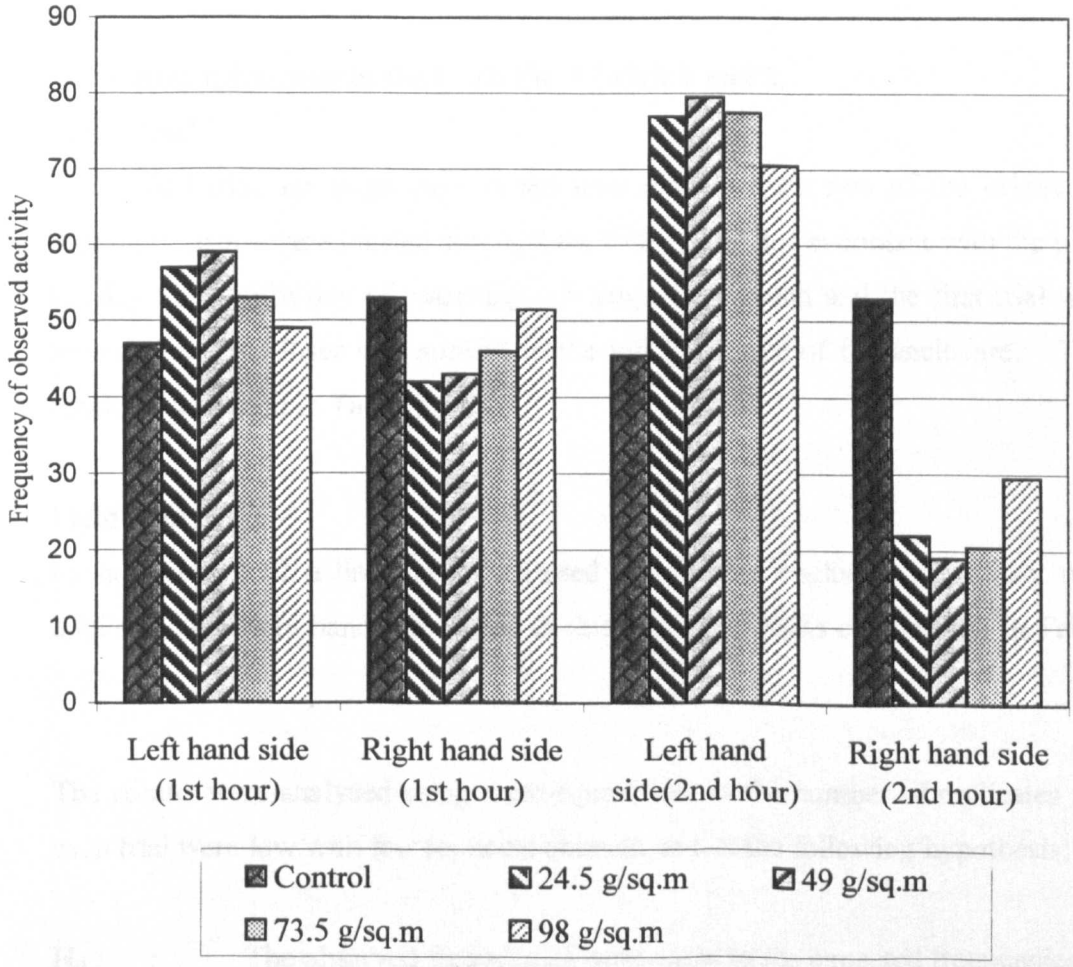


Table 7.3 Results of Kruskal-Wallis analysis of the avoidance data.

Fertiliser exposure group. (g/sq.m as granules in brackets.)	**Kruskal-Wallis test statistic (p values with df=3 for each group) for between period analysis**
Control (0)	**0.814**
50% (24.5)	0.014
100% (49)	0.039
150% (73.5)	0.0025
200% (98)	0.019

No relationship was detected between body mass and sex of individuals and the severity of the responses recorded.

7.3.3 Avoidance behaviour in the field: Field Trials 1 and 2.

Field Trial 1

In the first trial, no frogs were found until day 3, when two of the originally introduced frogs were located amongst the foliage and not in contact with the soil. On day 8, the fifth day of searching, no frogs were found and the first trial was terminated. Fertiliser was applied to the left hand side of the enclosure. The results can be seen in Table 7.4

Field trial No. 2

In the second trial, a finer mesh was used to cover the enclosure. Fertiliser was applied to the right hand side of the enclosure. The results can be seen in Table 7.5.

The results were analysed using a chi-square test as the number of replicates for each trial were low with few replicate animals, to test the following hypothesis;

H_0 : The observed frequencies were equal to the expected frequencies.

H_1 : The observed frequencies were not equal to the expected frequencies.

Table 7.4 Results of Avoidance Field Trial No.1 (Fertiliser applied LHS).

Enclosure half	1	2	3	4	5	6	7	8	Totals
Left Half	1	0	-	-	0	0	-	0	1
Right Half	2	3	-	-	2	2	-	0	9

Table 7.5 Results of Avoidance Field Trial No. 2 (fertiliser applied RHS).

Enclosure half	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Totals
Left half	1	2	-	-	-	1	3	2	-	3	-	-	3	1	1	2	-	-	19
Right Half	2	1	-	-	-	1	0	1	-	0	-	-	0	2	1	0	-	-	8

In field trial 1, after applying Yates Correction, the chi-square value of 22.06 exceeded the critical values at both the 1% and 5% significance levels. This suggests that the difference in the observed frequencies of frogs in each half of the enclosure was not a random occurrence. By observation of the data, 10 observations of frogs were recorded during the first field trial, 90% of observations were of frogs residing in the untreated side of the enclosure. The presence of ammonium nitrate appeared to be having a significant effect on frog location within the enclosure. Although it is possible that individual frogs were predated in this trial, it does illustrate that even with a small number of individuals, it is still possible to demonstrate that the observations recorded in the laboratory can be extrapolated into a simulated field situation.

In field trial 2, a small number of frogs were introduced into the enclosure, but were monitored over a longer period. After applying the Yates correction with 1 degree of freedom, the Chi-square value of 23.23 exceeded the 1 and 5% critical values. The effect of these results was to reject the H_0 hypothesis and accept the H_1 hypothesis. This suggests that a significant difference does exist between the observed and expected frequencies, and that this difference was not a random occurrence and could not be explained by sampling error. By inspecting the

limited data set, there is a significant difference between the number of frogs observed over the duration of the study, residing in the untreated side of the enclosure (70%).

The results of both trials using limited data sets do show that under field conditions ammonium nitrate can significantly modify individual preference behaviour and thereby alter habitat selection.

7.4 Discussion

From the results, activity levels and avoidance behaviour in frogs were modified following short periods of exposure to moderate levels of ammonium nitrate fertiliser under laboratory conditions. Individual activity levels monitored under laboratory conditions were modified following an acute exposure to moderate to high levels of ammonium nitrate fertiliser. At relatively high levels of fertiliser (>50% SFAR) behaviour was such that individuals would become motionless with elevated lung ventilation activity. An increase in the concentration of ammonium nitrate fertiliser above 50% exposure led to elevated levels of activity observed over the test period. Individual frog preference behaviour was shown to be modified following an acute exposure to relatively low levels of fertiliser exposure condition under laboratory conditions. Significant differences were established between the time spent in unexposed (control) or exposed halves of a preference chamber. At 50% (24.5 g/sq.m) exposure concentration, frogs tended to spend up to ¾ of their time in the unexposed half of the test chamber. This followed for each level of fertiliser exposure with a slightly longer proportion of time spent in exposed halves of the test chamber at the higher exposure concentrations. This may be explained by individuals being affected to such an extent that they were unable to actively move away from the exposed half of the chamber. If the individual was able to minimise body contact within the exposed test chamber, there still existed a lag affect of fertiliser adhered to the skin of the frog.

If a frog were to happen across a pool or a surplus pile (as was seen on many occasions in the field whilst sampling, resulting from spillage whilst filling the hopper on the tractor) then the affects would be severe, and would probably result in death. It was noted that in areas where a surplus of ammonium nitrate was seen, up to a week later, the granules had dissolved and leached into the soil, and left the area devoid of grass. If the individual frog was to land on granules, the initial response would be to jump away from the source of stress. Given the moist

nature of frog skin, it is likely that granules may become stuck to the skin, leading to a localised affect.

Field trials No. 1 and No. 2

The results, although not based on an extensive dataset, do illustrate that a potential does exist for frogs avoidance behaviour in the field to be modified, with adults being locating in the halves of the simulated wheat crops to which ammonium nitrate fertiliser solution had not been applied.

CHAPTER EIGHT

PRODUCTIVITY IN ADULT COMMON FROGS '*Rana temporaria*'

CHAPTER 8

PRODUCTIVITY IN ADULT COMMON FROGS *'Rana temporaria'*

8.1 INTRODUCTION

A biological indicator species may be defined as one that gives an early warning of the degradation of an ecosystem through its disappearance or disturbance within its natural habitat (Lawrence 1995). Warnings may manifest as physical (disappearance or physical damage), physiological or biochemical (a biomarker) response which may be quantified following exposure to a pollutant. Peakhall (1992) states that indicator species have a higher degree of susceptibility to environmental pollution compared with other organisms present within a habitat. Because of species specific responses, pollutants may be detected by selecting the susceptible indicator species (Peakhall 1992). The biomarker responses are such that they may be directly related to levels of exposure, and are used to implement control measures to reduce the potential environmental impact of a particular pollutant. A biochemical biomarker is not of particular interest during this research, as detection of these would involve destructive assays. Instead, the intention was to investigate physiological responses such as respiration and growth. These will be combined and used as an assessment of individual fitness. Using energetic equations, a common frog's 'Scope for Growth' could be assessed, and may be linked to an individuals fitness, and be related to population success (Maltby 1990).

8.1.1 Amphibian energetics

Amphibians have a low annual use of energy (Pough 1983). Most amphibians do however engage in increased levels of activity that require higher demands on energy expenditure such as avoiding predation, (Blem *et al.* 1986) and migration to breeding sites requiring sustained locomotion (Magnusson *et al.* 1988).

For all individuals, there are underlying minimum rates of energy expenditure, directly related to the maintenance of normal resting body function. This is the basal metabolic rate, and corresponds to the minimal energetic cost for body function and maintenance

(Pough 1983). In order to study energy budgets in amphibians, constant temperatures must be maintained as temperature variation affects levels of energy expenditure, as seen in other ectotherms (Krogh 1904, Whitford 1973). To complicate matters further, the time of day also affects the energy budget independent of temperature, (Taigen & Pough 1981).

Basal metabolic rate (BMR) is assessed by measured oxygen consumption (VO_2) of resting organisms. To reduce the effects of temperature and time of day on the BMR, it is important to measure VO_2 levels under controlled laboratory conditions. BMR has been calculated for common frogs, at differing temperatures. Kayser (1940) found that at rest and at 10°C, a 38g (wet body mass adult common frog) has a VO_2 of 0.814 ml/hr. In contrast with similar mass frogs, at 15, 15.1, 19 and 19.2°C; VO_2 consumption was measured at 2.60 (Vernon 1897), 0.49 (Krogh 1916), 1.58 (Kayser 1940) and 2.72 (Bohr 1900) ml/hr respectively. At an ambient temperature of 24°C, VO_2 consumption was measured at 3.46 ml/hr (Dolk & Postma 1927). This gives a range of VO_2 between 0.49 to 3.46 ml/hr at temperatures between 10 and 25°C, for adult frogs of approximate mean mass of 38 g. Kasbohm (1965) measured a VO_2 of 4.3 ml/hr for a 42g frog at 23°C. In comparison, a VO_2 of 0.352 ml/hr was found for a 30g frog at 14°C (Bastert 1929).

Body mass in combination with body length has been used as an indictor of individual and evolutionary fitness (body condition index). Animal fitness (Jakobson et al 1995) at any one time can be used as an indicator of historical foraging success and as a measure of individual ability to deal with stress. This may indirectly or directly influence reproductive success, ultimately having positive or detrimental impacts on the long-term fitness of individuals.

This chapter aims to develop a non-invasive technique for determining the respiratory efficiency of common frog (*Rana temporaria*) so that potentially, it could be used as a physiological indicator of stress in the field. This chapter follows the development of a technique that can provide information on the sub-lethal impact of ammonium nitrate fertiliser on respiration. However, as will be described, the initial design and

construction of the respirometer highlighted a problem when using common frogs in large respirometry vessels. The basal metabolic rate for common frogs is extremely low, and levels of oxygen consumption are therefore difficult to measure. The first respirometer design gave no results. The subsequent design used smaller respirometer chambers and gave a better indication of an effect following periods of prolonged exposure to ammonium nitrate at low concentrations.

Over a prolonged period of exposure in the laboratory, productivity was also assessed, by determining the intake and output of energy in individual frogs. By using bomb calorimetry, it was possible to measure the food conversion efficiency of frogs.

8.1.2 Scope for growth.

The 'Scope for Growth assay' is an integrative physiological indicator (Baillieul *et al.* 1996) used to investigate energy budgets and potentially the impacts of pollutants on individuals organisms. Common frogs are at risk from ammonium nitrate fertiliser in the environment (Hilton-Brown & Oldham 1991; Oldham *et al.*, 1997). Exposure to ammonium nitrate fertiliser, either directly (contact with whole granules) or indirectly, (absorption of fertiliser in solution across the integument or via the diet), has the potential to reduce individual fitness. The skin of amphibians is permeable to many different chemicals (Savage 1951). By monitoring physiological responses before, during and after exposure to a pollutant, it is possible to quantify the effects of an individual pollutant on individual fitness. The Scope for Growth assay combines individual respiratory efficiency with individual food conversion efficiency, giving a value that may give a reliable indicator of individual fitness.

Mathematically, Scope for Growth is directly related to productivity (**P**) in the balanced energy equation of Winberg (1960). The energy equation is shown below:

$$\mathbf{C - F = A = R + U + P}$$

Where:

A :Energy absorbed from the diet

C :Energy consumed.

P :Energy allocated into productivity.

R :Energy metabolised through respiration.

U :Energy loss from absorbed energy as excretory products

F :Energy loss as faeces.

The greater the value of P, the greater an individual's fitness will be, as potentially, this will place that individual at a selective advantage when it comes to reproduce, being able to incorporate more productive energy into the production of healthy gametes.

To simplify matters, the Scope for Growth equation may be rewritten as:

Productivity = C-R-F

Where;

C = Energy in consumed food.

R = Energy used in respiration.

F = Energy lost to waste products and therefore not assimilated.

The difference between the energy absorbed from the diet, and the energy lost to productivity, respiration and excretion.

8.1.3 Mitigating Circumstances in the application of the Scope for Growth Assay

The energy budget of amphibians including the common frog is extremely low when compared to mammals. Some species of amphibian temporarily depart from acquiring energy from diet and attaining mass, or maintaining homeostatic equilibrium periods in their lives.

8.2 METHODS

The first technique was designed to assess the effect of the fertiliser on individual respiratory efficiency, and the second to measure individual food conversion efficiency. Adult common frogs were exposed to ammonium nitrate in the laboratory. They were given known amounts of food (of known calorific value) and faecal pellets were collected. The amount of energy in food items presented to the frogs and the energy contained in faecal pellets was measured using bomb calorimetry. A respirometer was developed to measure VO_2 – basal metabolic rates. The results of these two components were combined to assess individual Scope for Growth.

8.2.1 Holding conditions

Common frog adults collected from Coleorton, North West Leicestershire, were transferred to the testing facility at Scraptoft Campus in Leicester, where they were sexed and their masses determined. A group of nine frogs was divided into three groups of three animals and placed into pre-exposure holding tanks (6 x 25-litre all glass aquariums) for a five day acclimation period. Handling during this time was kept to a minimum. The tanks were supplied with a refuge and a uniform blotting paper substrate soaked in artificial pond water to maintain moisture levels. The testing facility was maintained at 16 ± 1 °C, with approximately 55% ± 5% humidity. All frogs were fed *ad libitum* with house crickets *'Acheta domestica'*. Moisture levels were maintained by spraying aged tap water into the tanks on a daily basis if the blotting paper appeared dry. At least once during the first week, the blotting paper substrate was renewed and re-wetted using artificial pond water. All tanks were covered with glass lids to reduce moisture loss from evaporation and prevent the loss of frogs.

The frogs were then divided into nine individual test tanks and held for an additional five day period. Each tank was supplied with a refuge and uniform blotting paper substrate with moisture levels maintained in a similar manner to the stock holding tanks. During the five day isolation period, all frogs were starved to void stomach contents before the start of the test.

8.2.2 Testing regime and Test Concentrations

Frogs were exposed to ammonium nitrate fertiliser with substrates and test solutions being renewed approximately every 96 hours (four 12hr light : 12hr dark cycles). Two nominal exposure concentrations were selected with a control.

Test concentrations were determined according to the following rationale;
If a concentration equivalent to the national average field application rate of 15 g/m^2 as NO^-_3 N was selected, there would be greater than 50% mortality as the established LC_{50} value for adult frogs was 3.6 g/m^2. In the field, it is unlikely that individual frogs would come into direct contact with ammonium nitrate granules at such a concentration, and if so, this would represent a worst case scenario. Factors such as diurnal application of the fertiliser, the non-homogenous nature of the soil topography and the relatively quick dissolution rate of ammonium nitrate into soil and / or surface water, reduces the concentrations to which individual frogs would be exposed. Experiments in the field (Hill, 1996) have predicted that at least 50% of ammonium nitrate fertiliser granules applied to a developing crop are lost to the uneven structure of the soil surface layer. The LC_{50} concentration of 3.6 g/m^2 NO^-_3 N was used as the highest concentration to be considered. In the trials a control, a low and a high level of exposure were used. The concentrations chosen therefore are based on levels lower than established LC_{50} values. The values were 1 and 3 mg/l of ammonium nitrate fertiliser applied as NO^-_3 N. Low levels of exposure were selected to minimise mortality rates during the testing. Due to a limited number of frogs being available, and because a reliable LC_{50} was already established, a rangefinding test was not undertaken. As the LC_{50} data were available, this was used as a starting point with the individuals being exposed to solutions instead of granules. At each treatment concentration, three replicates of three frogs with each frog being > 10 g and <50g body mass. The surface area of the substrate between tanks was uniform at 28 x 19cm, equivalent to 0.53 m^2. The exposure concentrations were prepared adding the required amount of ammonium nitrate to each saturated blotting paper (ca. 50-60 mls of artificial pond water, added to 300g/m^2 blotting paper). To simulate the required exposure conditions at 1 and 3 g/m^2, 683.7 and 2.051mg were added to test vessels. Each amount was corrected for the NO^-_3 component of the fertiliser by multiplying by 1.29. (the molecular weight of NH_4NO_3 divided by the

molecular weight of NO^-_3 = 80/62 = 1.29). The concentration of fertiliser applied was not checked due to the nature of the application. On a saturated substrate, the fertiliser was presented in a more homogenous method, compared to exposing frogs to granules over the same surface area. After application, the test vessels were left to equilibrate for 60 minutes, which allowed the granules sufficient time to dissolve into solution (Hill 1996). In the control treatment, artificial pond water was used.

8.2.3 Frog Feeding regime

All frogs were fed *ad libitum* with house crickets (*Acheta domestica*) at a daily rate that was equivalent to 6% of individual frog body mass.

8.2.4 Oxygen consumption

The nine exposed or control frogs were removed from the continuous exposure tanks on five occasions over the 37 day exposure period. Measuring oxygen consumption is a non-invasive method of establishing impacts on the metabolic systems of individuals placed in stressful situations. As the amount of energy required to metabolise a molecule of oxygen is known, it is possible to quantify how much energy would be utilised under stressful situations. A minimum requirement for metabolism is the basal metabolic rate (BMR), which can be established by measuring oxygen consumption. The BMR is calculated as the minimum metabolic effort or rate required for survival. Individual frogs were removed from their exposure tanks and weighed using a flat pan balance (1-decimal place). The frog was then transferred into a constant volume differential respirometer for a 2 hour testing period. Each chamber was sealed using vacuum grease applied at the junction of a large rubber bung and the testing chamber (jar), and open to air via an access tube inserted through the bung. The animal was left undisturbed in the chamber for approximately 10-15 minutes to acclimate to conditions. After this time, the air access tube was closed and voltage readings generated by an ultra low-pressure transducer were recorded at 5 minute intervals for the first hour and then every 10 minutes for the second hour. Before the frog entered the chamber, a calibration curve was constructed (section 8.2.9). The voltage readings from the pressure transducer are converted to volumes of oxygen, relating directly to the drop in the internal pressure within the respiratory chambers during a test as the frog consumes oxygen.

Corresponding voltages may then be converted to microlitres of oxygen per gramme of body mass.

8.2.4 Food conversion efficiency

From the end of the starvation period at the end of week two after collection from the field, all faecal pellets found in the test vessels were collected and dried to constant mass over 24 hours at 60°C. Pellets were then stored in air tight containers, labelled and frozen at *ca.* -18°C. This was repeated for the duration of the test. The calorific value of the food prey items offered to the frogs was established by measuring the calorific value of a size range of different instar domestic crickets using a bomb calorimeter. The calorific value of all faecal pellets collected during the 37 day duration was established using a bomb calorimeter.

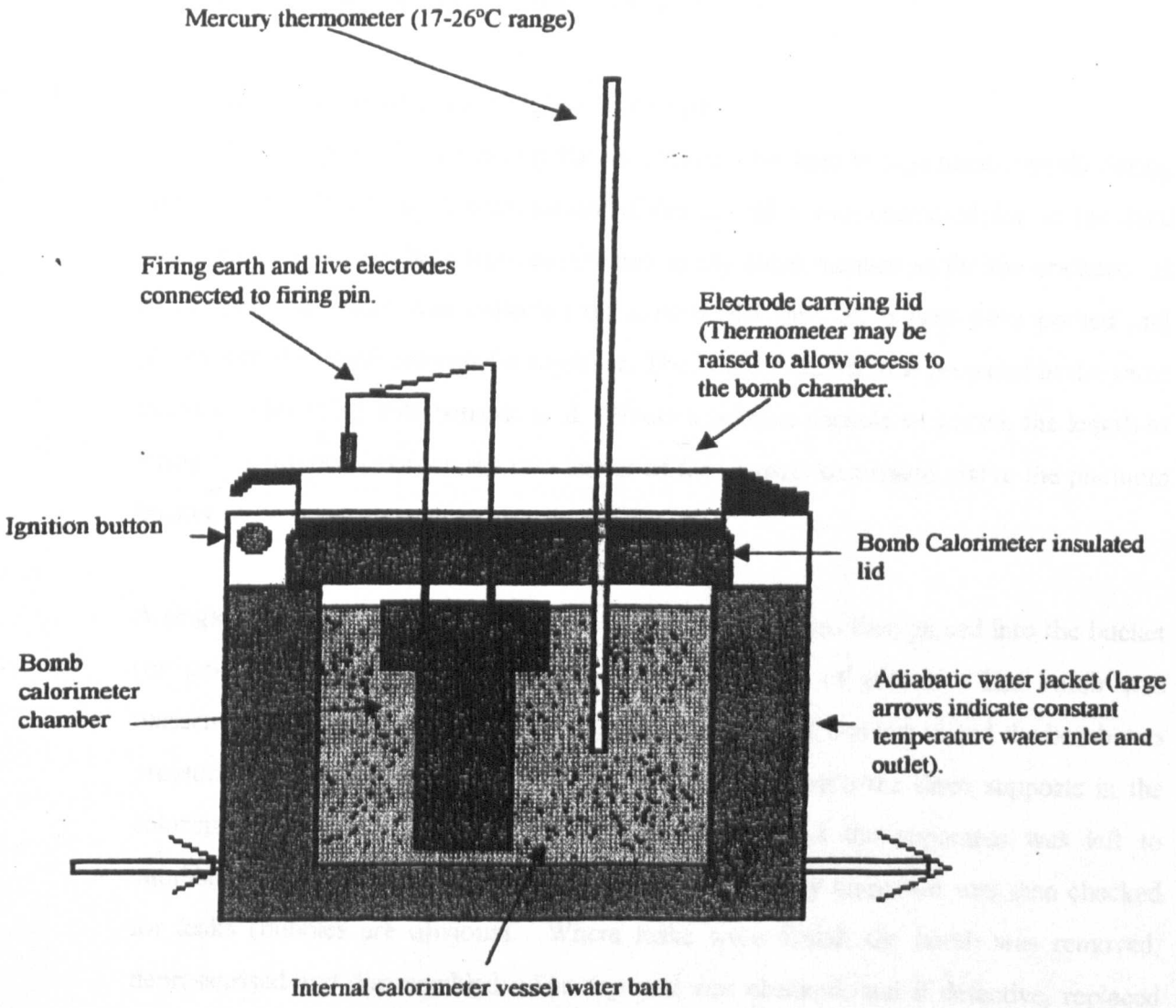
8.2.5 Bomb calorimetry

Crickets were killed using carbon dioxide overdose. They were immediately weighed (wet weight) and measured (head to posterior tip of abdomen), dried overnight at 60°C until a constant mass was achieved, stored in air-tight containers and frozen at *ca.*-18°C until required for analysis. The faecal pellets collected during the study were treated similarly

Heats of combustion were determined using a bomb calorimeter (Fig 8.1). The samples of dry cricket or faecal pellet, were ignited in an O_2 rich environment in a stainless steel calorimeter bomb vessel. The vessel was immersed into an adiabatically temperature controlled water jacket inside the stainless calorimeter vessel. The internal vessel has a highly polished internal surface of stainless steel allowing good heat conductance. Test samples were secured inside the bomb chamber, inserted into the water bath and ignited electronically by heating a nichrome wire circuit. The ignited sample burned freely in the O_2 rich environment resulting in a change in temperature in the external water bath of the bomb calorimetry apparatus. The temperature differential recorded by mercury thermometer (mercury thermometer filled with nitrogen BS 593), between the pre-ignition start of the test and the point at which the temperature ceases to increase, was

used in conjunction with the specific heat capacity of the calorimeter. This established the heat of combustion of the test samples.

Figure 8.1 Bomb calorimetry apparatus used for establishing the heats of combustion for house crickets and faecal pellets.



The bomb calorimeter was calibrated using a known standard to establish its specific heat capacity. This was achieved by burning a 1g pellet of benzoic acid for which the heat of combustion was accurately known. Each calibration followed the same procedure, with calibration occurring each day of use.

8.2.6 Determining the heat of combustion of samples

Particulate samples of dry faecal pellet or cricket were held in a gelatine capsule during combustion. The heat of combustion of the capsules was corrected for in the final calculations. Faecal pellets were combusted in the same manner as for the crickets. If more than one pellet was collected on a particular day, all pellets were pooled and placed into the bomb calorimeter together. The bomb chamber was prepared in the same manner as for firing with benzoic acid. Where a gelatine capsule was used, the length of string was trapped between the two halves of the capsule suspended above the platinum bucket.

A single cricket or single (or daily pooled) faecal sample was then placed into the bucket (or gelatine capsule) covering the ends of the strand of cotton. The bomb was reassembled, tightening the cap by hand. The firing circuit was tested and the bomb was pressurised with oxygen. The bomb was then lowered onto the three supports in the calorimeter vessel. The firing circuit was checked and the apparatus was left to equilibrate 15 minutes. The bomb now almost completely immersed was then checked for leaks (bubbles are obvious). Where leaks were found, the bomb was removed, depressurised and disassembled. The cap seal was checked, and if defective, replaced and the bomb chamber was reassembled. The bomb was pressurised and checked as before and placed back into the apparatus. The jacket lid was then lowered ensuring that the firing electrode made good contact with the bomb. Depressing the circuit testing button tested this. If the switch lit up, then a good electrical was made. The apparatus was allowed again to acclimatise. The temperature was recorded as the initial temperature in degrees Kelvin. The firing switch was then depressed for 5 seconds and released. Successful firing was indicated by i) no light on pressing the circuit testing button, and ii) the bead of mercury in the accurate thermometer rising. The temperature after 10 minutes or when a constant value was obtained on the thermometer was recorded as the final temperature in degrees Kelvin. Following the test, the power was

turned off and the jacket lid was raised. The bomb was removed from the calorimeter vessel and depressurised carefully. The bomb was then disassembled and cleaned thoroughly using a paper towel. Spent firing wire was removed from the electrodes, and the bomb was then prepared for the next sample. The cooling coil water was increased to a rapid flow to lower the jacket temperature ready for the next test.

The temperature differences were used to calculate the amount of energy in joules released from each sample as it combusted using the following equations.
The specific heat capacity of the calorimeter was required Equation 1;

$$(\alpha) = (26452.2 * (\mathbf{b})) / (\mathbf{c})$$

Heat capacity of the calorimeter = (α) J K^{-1}

Heat of combustion for benzoic acid = 26452.2 J g^{-1}

Mass (g) of benzoic acid pellet = **(b)**

Temperature difference (K) = **(c)**

The heat capacity of the calorimeter (α) was used to establish the combustion energy of the test samples using Equation 2;

$$(\beta) = ((\alpha) * (c)) / (y)$$

Combustion energy of a sample (β) J g^{-1}

Heat capacity of calorimeter = (α)

Sample dry mass (g) = **(y)**

Temperature difference = **(c)**

Once the combustion energy of the sample has been established, this was converted into calories using the Equation 3,

To convert joules to calories, divide joules by 4.184 = 1 calorie.

8.2.7 Respirometry testing apparatus and procedure

The respirometer design selected for construction was that of a constant volume differential respirometer. The respirometer was designed to quantify pressure change

between linked chambers, converting it to a voltage. The volume removed from the balancing chamber was directly equal to the volume of air respired within the frog's chamber. As the frog respires, air was assimilated into the frog from the chamber. Oxygen was absorbed into the circulation of the frog, and carbon dioxide was released as the by product of respiration, along with some water vapour and inert gases. Residual carbon dioxide in the chamber at the start of the acclimation period was absorbed by a chemical carbon dioxide scavenger present in the animal chamber, leaving a gas volume deficit in the frog chamber. The unoccupied chamber was securely sealed, maintaining a constant volume at all times. As the pressure in the occupied chamber dropped there was net movement of air pressure towards the frog chamber to balance the pressures between the two chambers, connected via an ultra low differential pressure transducer. As the pressures attempt to equalise between the two chambers, a pressure gradient was formed between the two chambers towards the occupied chamber. The pressure was detected by the sensitive diaphragm in the ultra low differential pressure transducer, and displayed as a voltage on a suitable meter. By using a calibration curve for the transducer, this voltage may be converted to a volume, which in this case given that a carbon dioxide scavenger is in place, may be directly related to the volume of oxygen being respired by the frog.

The respirometer consisted of an Ultra low differential pressure transducer, (RS Components) (Fig 8.1) connected in series to two glass respirometer chambers (P1 and P2) which were equal in volume (Fig 8.2). Connections to the transducer, were made using 2x 250mm lengths of 5mm internal diameter Tygon rubber tubing. Volumes for all connecting tubing were calculated and corrected for in the final analysis. Pressure changes across the transducer during each test were displayed as a voltage using a digital display (Pantec digital multitester meter set to display voltage (Fig 8.3)). Voltage supply to the test system was supplied via a 4 amp / 16 volt constant power supply, with a working voltage range of 3-16 volts. The voltage of the test system was maintained at 6 volts (within the safe working limits of the transducer (4 - 12 volts)) with a negligible fluctuation over the duration of each test.

The volume of each chamber was established by filling completely with tap water and recording the volume. Each chamber was approximately 1156.9mls (± 4ml) in volume. Attached to the inside wall of each chamber by means of aquarium sealer, were perforated 15ml plastic test tubes. Each tube (one in each chamber) was filled with 3 grammes of Carbosorb (a carbon dioxide scavenger) with granule sizes larger than the perforations in the test tubes. The chambers were sealed with large rubber bungs and labelled **P1** (animal chamber bung) and **P2** (balancing chamber bung) (Fig 8.3) and then smeared in vacuum grease, ensuring that each chamber was airtight.

Two 6mm holes were drilled through bung **P1**, equidistant from a central point. One hole accommodated a 70mm x 5mm internal diameter glass tube. The tube was connected to the rubber tubing attached to the **P1** port on the transducer (Fig 8.3). The second hole initially accommodated a sintered glass two-way burette tap that was later replaced by a glass 1ml syringe.

One 6mm hole was drilled through the **P2** bung, to accommodate a 70mm x 5mm internal diameter glass tube. This was connected to the Tygon rubber tubing attached to the **P2** (Fig 8.3) port of the transducer. The power supply and voltage connections were made using a temporary circuit board construction, shown in Figure 8.3.

For the test system to work correctly, both chambers were held at the same constant temperatures for the duration of the test. Temperature control was achieved by submerging both chambers in a thermostatically controlled circulating water bath, consisting of a plastic 10-litre aquarium fed by a thermostatically controlled circulating water pump. Temperature was maintained at a constant 16 ± 1°C This was measured using a digital thermometer with external probe inserted into the chambers through the bungs, and sealed in place using silicon sealer, covered in vacuum grease.

The test system, with all components may be seen in Plate 8.4.

8.2.8 Principles of ultra low differential pressure transducer operation

The pressure transducer operates by detecting the electrical difference between the pressure at the **P1** port and the **P2** port of the transducer that are separated by a sensitive 'electronic' diaphragm.

In this case, the ports were attached to the respective respirometry chambers (**P1 & P2**) both being held under conditions of equal temperature and pressure and not affected by changes in external pressure. Any drop in the internal pressure of chamber **P1** would lead to a change in the voltage passing across the electronic diaphragm of the transducer. The positive pressure from the **P2** chamber forms a pressure gradient towards the **P1** chamber, to equal the pressure between the two chambers. This pressure change detected by the transducer was converted into a voltage and displayed on the multitester meter display.

The stated manufacturer's quoted accuracy of the transducer was ± 4% between 1.6 and 13.7 volts. Beyond these values would result in increased voltage resistance and leading to fluctuations and inaccurate readings. The accurate and safe working range of the transducer was set to between 4 and 12 volts.

Plate 8.1 Ultra–low differential pressure transducer used in the adult common frog respirometer testing apparatus.

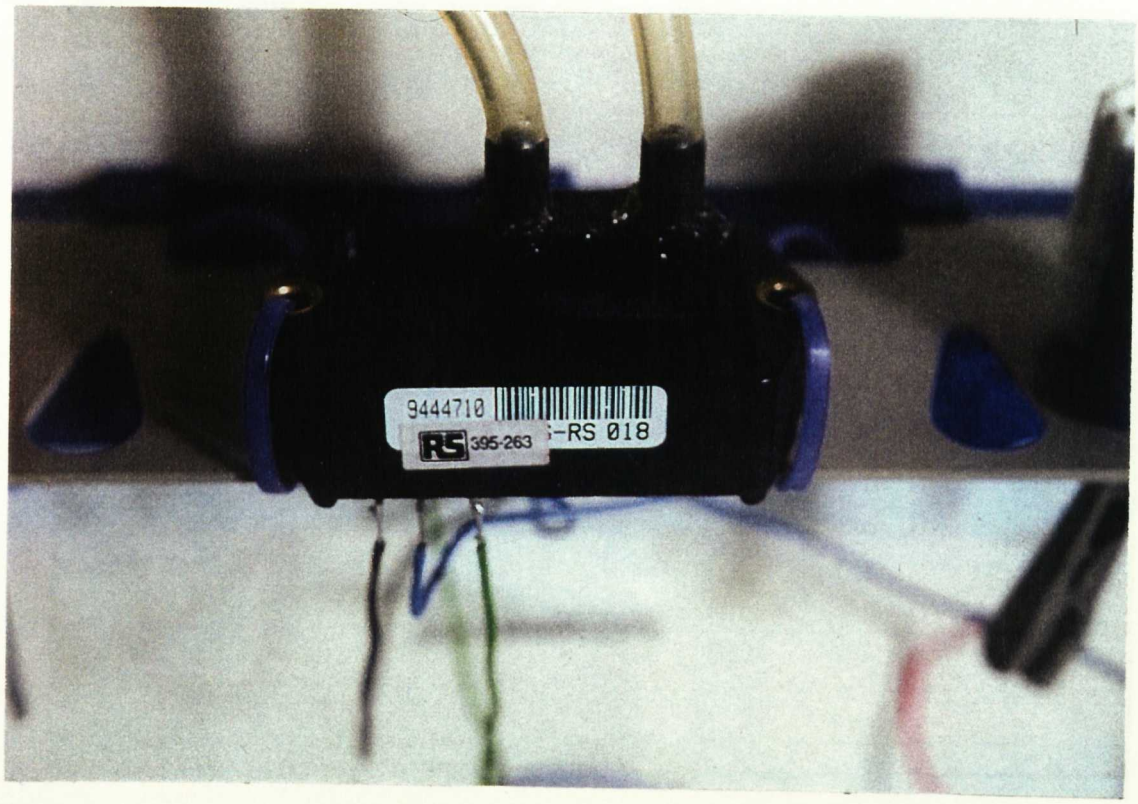


Plate 8.2 Adult common frog respirometer testing chambers (P1 & P2) in water bath (E).

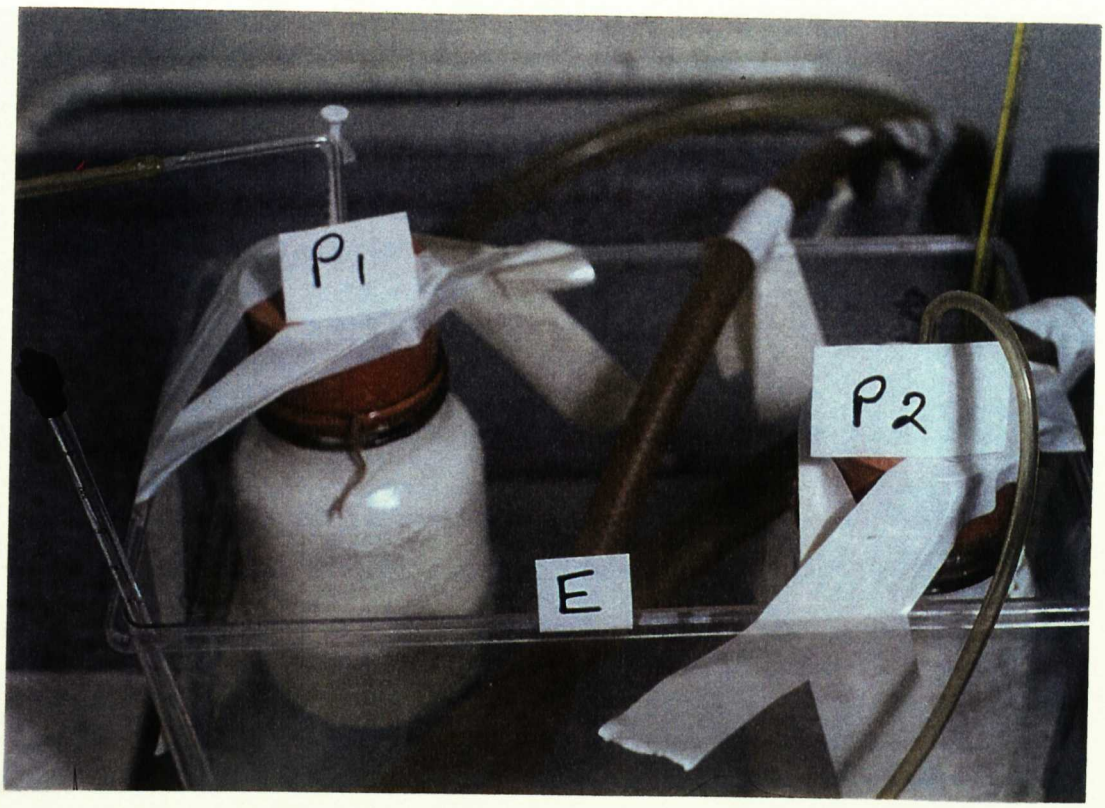


Plate 8.3 Ultra-low differential pressure transducer circuitry showing prototype circuit board (B), ports (P1 & P2) connecting transducer to test chambers, power supply connections and voltmeter display (D).

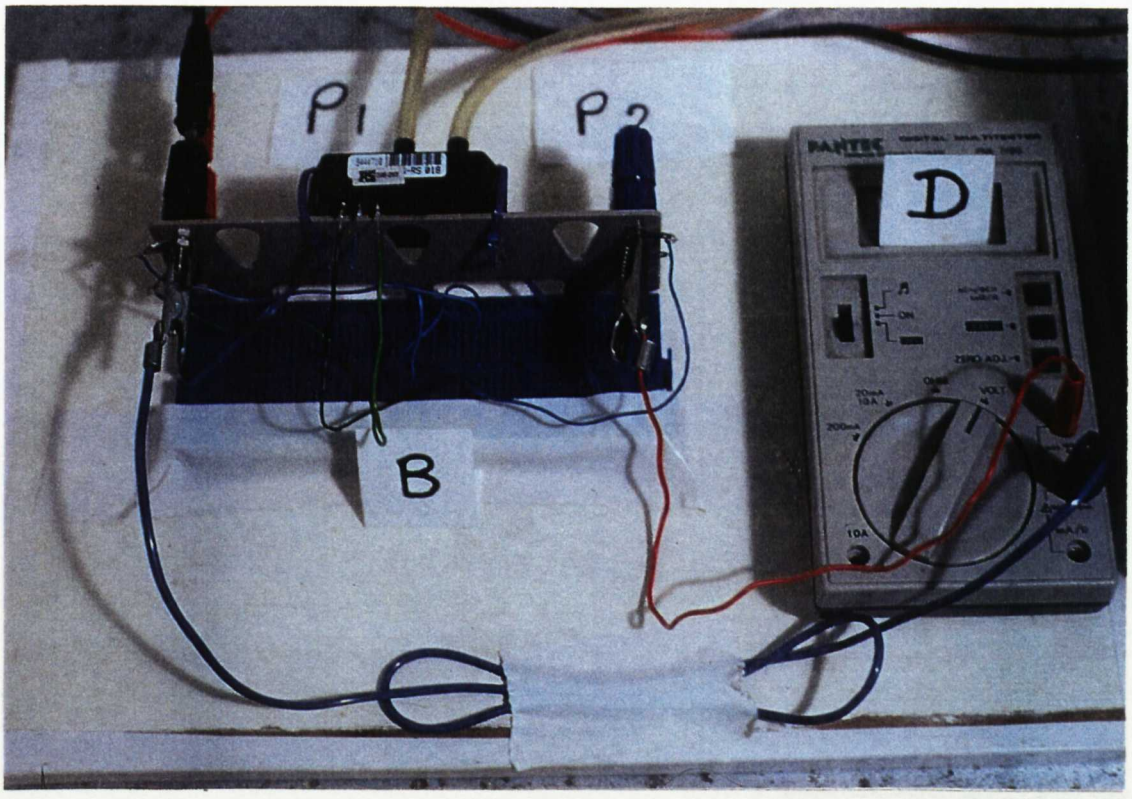
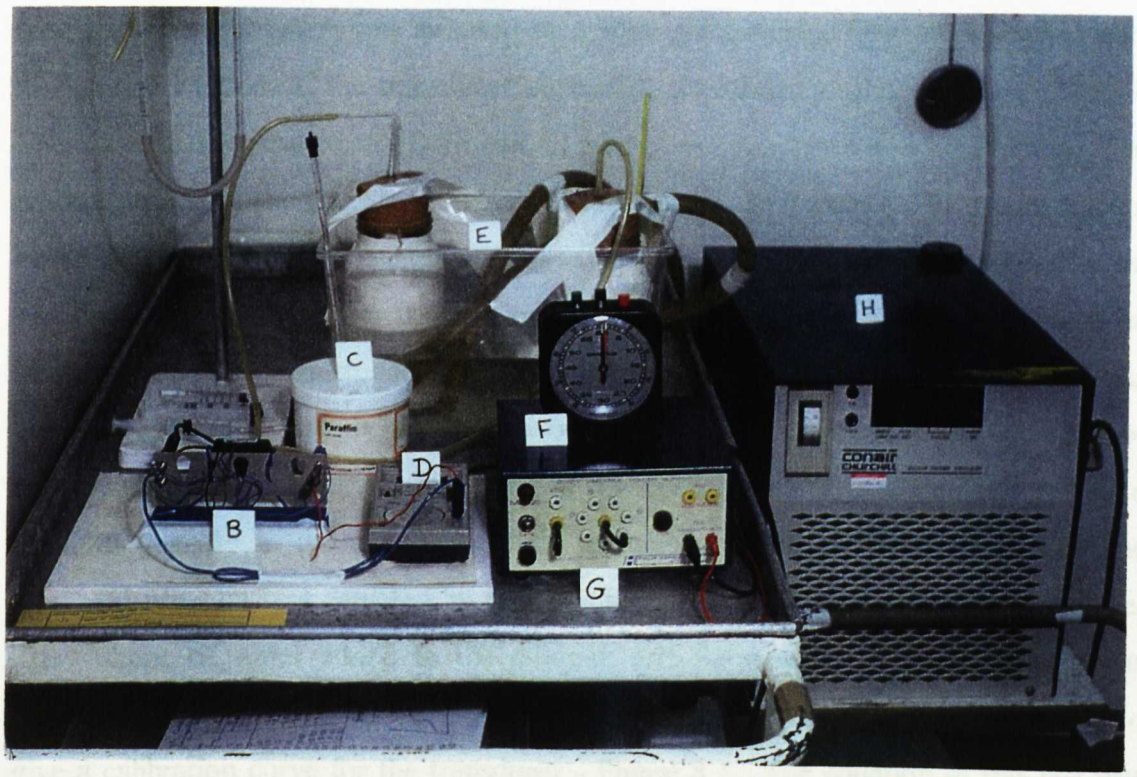


Plate 8.4 Respirometry testing apparatus showing ultra-low differential pressure transducer circuit board (B), voltmeter display (D), constant voltage power supply (G), stop clock (F), paraffin wax (C) for sealing testing chambers, water bath (E) containing the test chambers and the thermostatically controlled circulating water pump (H).



8.2.9 Calibration of the pressure transducer

A Warburg manometer with a capillary width of 1mm and a graduated length of 35cm marked in 'ml' was used to calibrate the transducer (Fig 8.1). The manometer was then filled with Anachem Respirometer fluid, which has low properties of adhesion to glass and a known density. The manometer was left to equilibrate to atmospheric conditions within the test facility for 2 hours. This also allowed time for any air bubbles to escape from the respirometer fluid. Following a 2hr equilibration period, a fine silicon capillary tubing (0.6mm diameter) was attached to a 50ml glass syringe filled with respirometer fluid. The P1 port of the transducer connected to voltmeter (the ultra low pressure transducer was connected to a 0-16 volt / 4 amp power supply set at 6 input volts). The working range of the transducer was between 3.6 and 13.7 volts. The water column height was corrected to 0 using a central valve at the mid-point between the two arms. The fine capillary tubing was passed into the remaining open arm to the meniscus of the fluid column. Respirometer fluid was added to the existing height (in 5mm additions using the graduated scale behind the manometer as a guide) of fluid in the open arm of the manometer. The addition of fluid in the open arm of the manometer led to a pressure increase in the air space of the column attached to the transducer. The increased pressure was detected by the transducer, and displayed as a voltage change. The volume of water added to the column, and the resulting voltage reading was recorded. The difference between the first and subsequent readings was then plotted to give a calibration curve for the transducer - Figure 8.1. The equation of this line was determined. Voltage readings could then be substituted into the equation and VO_2 respired by test frogs within the chamber calculated. The regression equation for the calibration curve was calculated and is shown on Figure 8.2. By rearranging the equation, the voltage was directly related to fluid volume detected by pressure change in the transducer. The linear equation, rearranged for x, where x = the height of fluid added to the manometer or equivalent to a volume change (pressure w) change across the transducer and y = the voltage recorded by transducer;

$$x = y - 0.0926 / 0.0961$$

A correlation coefficient was established between voltage and volume, giving a coefficient of 0.9992, which was > 0.95 and considered to be acceptable.

8.2.10 Determination of the oxygen consumption of adult frogs

Both chambers were acclimated to test conditions for at least one hour before the start of the test. This ensured that the test system was air tight with no leaks. Individual frogs would be held in respirometer chamber P1, for a total of 150 minutes during testing. A frog was selected from the testing vessels, according to a predetermined test regimen, and weighed before being placed into the **P1** chamber. To reduce the amount of stress to which the frogs were exposed, each chamber was blacked out and covered using opaque waterproof tape around the outside. This process was routinely repeated for the balancing chamber. The test chamber **P1** and the balancing chamber **P2** were held in the thermostatically circulating water bath, held at 16°C. Frogs were given a 30 minute equilibration period to acclimate to test conditions within the chamber. In this period, the two-way tap to the chamber was open for 10 minutes, after which it was closed completely, making the system airtight. After a further 20 minutes, the voltage passing through the transducer was recorded as a time zero reading. Voltage was recorded every 5 minutes over the subsequent, first 30minute period. For the remaining 90 minutes, voltage readings were recorded at 10 minute intervals.

To establish the volume of oxygen consumed the following formula was used;

$$\Delta N = (k)\ V\ \Delta P/T \qquad \text{(Elliot \& Davison 1975)}$$

Where,

ΔN = Number of oxygen molecules respired by the frog.

(k) = Number of oxygen molecules in 1ml of air (1.804×10^{19})

V = Volume in mls of chamber **P1**.

ΔP = Pressure change measured in inches of water (1 inch = 24.6mm)

T =Temperature of water bath measured in °Kelvin (273 + °C).

Worked example using Equation (1)

An adult male frog weighs 34 grammes and was placed into the **P1** chamber for 2hrs. The time zero voltage recorded by the pressure transducer was 6.67 volts. After the 2hr period, the voltage had dropped to 3.42 volts, a difference of 3.25 volts. Chamber volume = 1134.44ml. Bath temperature = 16°C. What was the number of oxygen

molecules consumed by the frog over that period, expressed as molecules of oxygen per 34g frog, and the volume of oxygen consumed per hour, expressed as ml/hr/gramme of frog?

From the transducer calibration, a change in voltage of 3.42 volts, corresponds to change in mm of fluid 167mm. = $\Delta P = 167 / 24.6$

$= 6.79$ inches.

The chamber volume was measured at 1134.44ml = V

Temperature of the water bath was 16°C, therefore in °K, this corresponds to 289°K=T.

1. The amount of oxygen available in the chamber is equivalent to ca. 21% of the chamber volume $= 1134.44 \times 0.21$

   $= 238.2$ ml O_2

2. Knowing $k = 1.804 \times 10^{19}$, 238.2 ml of oxygen

   $= 429.7 \times 10^{19}$ molecules of O_2

3. Pressure change equals 167mm, therefore;

   $= 167 / 24.6$

   $= 6.79$ inches of water.

Therefore, substituting the values into the ΔN equation,

$= 429.7 \times 10^{19} \times (6.79/289)$

$= 429.7 \times 10^{19} \times 0.023$

$= 9.88 \times 10^{19}$ molecules of oxygen consumed in 2 hours

$= 4.94 \times 10^{19}$ molecules of oxygen / hr

$= 2.74$ ml / O_2 / hr

Or Where frog mass =34g, $= 0.081$ ml / O_2 / g / hr

To convert oxygen uptake to energy consumption, an oxy-joule equivalent of 21 kJ/litre of oxygen was used (Elliot & Davison 1975).

Using this value, the amount of energy used by the frog in the example;

$= 2.74$ ml / O_2 / hr

$= 0.00274$ litres / O_2 / hr

Energy used $= 0.00274 \times 21$

$= 0.0573$ kJ of energy

= 57.3 joules of energy used in 2 hours

8.2.11 Testing regime

Each oxygen consumption determination test was 180 minutes (150minutes test + 30 minutes preparation time) duration. Initially, it was predicted that to test all frogs once would take 3 days. The start time for each test was staggered over three days, with random allocation of individual testing times (within a 9 hour period). The allocated time would provide sufficient time to carry out tests on three frogs per day, and allow sufficient time between tests to disassemble and reassemble the test system and check for leaks. The first respirometry test of all frogs corresponds to the baseline levels of respiratory activity, with no individuals being exposed to ammonium nitrate until after they had been tested once in the respirometer chamber. Animals were tested in order of concentration to reduce the risk of any animal becoming contaminated during a testing occasion, by the previous frog in the chamber.

8.3 RESULTS

8.3.1 Respirometry Tests.

Table 8.1 displays the voltages recorded for the calibration of the transducer, with Figure 8.1a showing the calibration curve for the transducer.

Over the 37 day exposure period, the VO_2 for each frog was measured using the respirometer on 5 occasions, to assess the chronic effect of ammonium nitrate on basal metabolic rate by recording (VO_2) volumes of oxygen consumed in 'ml / O_2 / hr'.

8.3.2 Productivity

The body mass of each frog was determined before each respirometer test. The frog held in plastic bucket was weighed on a flat pan balance and then transferred to the P1 chamber of the respirometer. Table 8.2 shows the change in body mass over the duration of the test and the difference between day 0 and day 37. Table 8.3 shows the mean body masses (g) per exposure group with the associated 95% confidence intervals.

Table 8.1A-E Calibration of Ultra low differential pressure transducer a using Warburg manometer at 19°C at 748mmHg atmospheric pressure at a starting voltage of 6 volts. This was taken as zero voltage, with the difference in voltage between the starting voltage and after each addition of manometer fluid being recorded.

A	Respirometer fluid volume added to manometer arm mm (ca = 0.1ml)													
Meter reading (V)	0	5	10	15	20	25	30	35	40	45	50	55	60	65
Actual reading	5.99	5.81	5.72	5.59	5.44	5.35	5.25	5.16	5.07	4.94	4.83	4.69	4.61	4.52
Difference	0	0.18	0.27	0.4	0.55	0.64	0.74	0.83	0.92	1.05	1.16	1.3	1.38	1.47

B	Respirometer fluid volume added to manometer arm mm (ca = 0.1ml)											
Meter reading (V)	70	75	80	85	90	95	100	105	110	115	120	125
Actual reading	4.38	4.3	4.23	4.13	4.01	3.95	3.85	3.75	3.67	3.56	3.45	3.36
Difference	1.61	1.69	1.76	1.86	1.98	2.04	2.14	2.24	2.32	2.43	2.54	2.63

C	Respirometer fluid volume added to manometer arm mm (ca = 0.1ml)											
Meter reading (V)	130	135	140	145	150	155	160	165	170	175	180	185
Actual reading	3.27	3.19	3.09	2.98	2.86	2.77	2.66	2.64	2.53	2.41	2.31	2.22
Difference	2.72	2.8	2.9	3.01	3.13	3.22	3.33	3.35	3.46	3.58	3.68	3.77

D	Respirometer fluid volume added to manometer arm mm (ca = 0.1ml)											
Meter reading (V)	190	195	200	205	210	215	220	225	230	235	240	245
Actual reading	2.11	2.02	1.94	1.83	1.76	1.66	1.56	1.48	1.39	1.31	1.24	1.16
Difference	3.88	3.97	4.05	4.16	4.23	4.33	4.43	4.51	4.6	4.68	4.75	4.83

E	Respirometer fluid volume added to manometer arm mm (ca = 0.1ml)											
Meter reading (V)	250	255	260	265	270	275	280	285	290	295	300	305
Actual reading	1.09	0.99	0.84	0.73	0.62	0.54	0.43	0.32	0.24	0.15	0.04	0
Difference	4.9	5	5.15	5.26	5.37	5.45	5.56	5.67	5.75	5.84	5.95	5.99

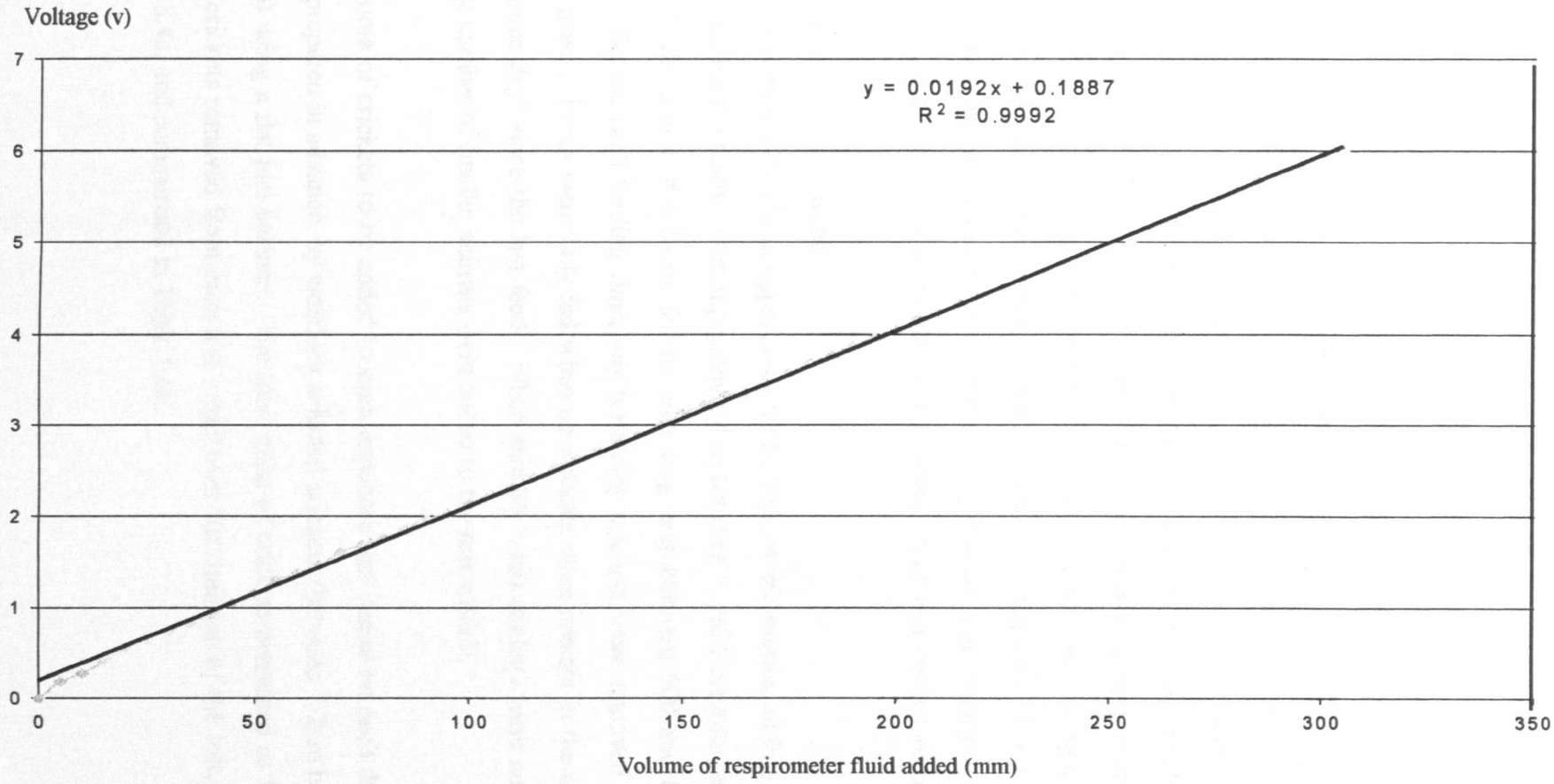
Table 8.2 Body mass (g) readings for all frogs (A, B & C) before being placed into the respiratory chamber on each occasion. Exposure groups shown are mg/l NO^-_3 N. (n=9)

Exposure Group	Body mass (g) recorded for each frog on each testing occasion (defined by day).					Difference (g) between Day 6 and Day 24
	6	10	14	24	37	
Control A	16.41	15.82	16.27	16.80	18.75	+2.34
Control B	19.26	21.3	20.34	21.43	23.96	+4.70
Control C	16.27	22.01	22.30	23.06	25.04	+5.94
1g/m^2 A	23.96	24.81	24.98	25.60	27.68	+4.02
1g/m^2 B	16.21	17.22	17.99	17.30	18.86	+2.06
1g/m^2 C	17.78	17.55	17.45	20.06	19.84	+2.06
3g/m^2 A	18.00	15.89	17.32	16.76	-	+1.24
3g/m^2 B	21.34	21.83	24.00	22.40	18.24	-3.10
3g/m^2 C	28.06	30.23	31.65	33.21	27.59	-0.47

Table 8.3 Mean body mass (g) of adult frogs at each exposure concentration (mg/l NO^-_3 N, with 95% confidence intervals.

Testing Occasion	Mean body mass (g) (n=3)			Body mass 95% Confidence interval (g (n=3; df = 2; t=4.303)		
	Control	1 g/m^2	3 g/ m^2	Control	1 g/ m^2	3 g/ m^2
1	17.31	19.32	22.47	± 2.05	± 3.19	± 3.57
2	19.71	19.86	22.47	± 2.90	± 3.26	± 4.23
3	19.64	20.14	24.32	± 2.76	± 3.23	± 4.22
4	20.43	20.99	24.12	± 2.84	± 3.24	± 4.56
5	22.58	22.13	22.92	± 2.89	± 3.47	± 4.05

Figure 8.1a Calibration curve for the Ultra Low differential Pressure Transducer at 748 mmHg atmospheric pressure and at 19°C, using a Warburg manometer using an initial input voltage of 6 volts through the transducer.



Body masses were recorded daily during each respirometer test for the duration (37 days) of the study. At the end of the study, the net change in body mass (g) was determined and is presented in Figure 8.3. The mean body mass on each test day during the exposure period is presented in Figure 8.2.

Analysis of variance revealed no significant differences between treatments of the mean body mass of individual frogs over the duration of the test ($f = 2.82$; $p = 0.071$ $df = 9$). Although a trend of reduced body mass existed with exposure concentration. Control frog body masses increased by more than 2g. One-way analysis of variance revealed no significant differences in the mean body masses recorded at the start and the end of the exposure period at 3 g/m^2. From the data (Table 8.2), it was apparent that body masses were significantly reduced at the highest exposure concentration. At $3g/m^2$, replicate B frog was found dead in the exposure tank on day 37 of the test. The two remaining frogs at the highest exposure group appeared quiescent and lethargic and were not actively feeding, with many of prey items remaining uneaten in the test vessels.

8.3.3 Consumption of food items

The adult common frog is an opportunist feeder and for this reason, all frogs were fed *ad libitum* during the study. This approximated to between 3 and 5 crickets per day. When required, the quota of crickets fed to each frog was between 500 and 800mg (fresh mass). Before each feeding time, any remaining crickets were removed and weighed (fresh mass). Frogs were only fed when no crickets were present in the test vessels or after three days since the last feed. When suitably sized crickets were not available, a greater number of smaller animals were added to the test vessels.

The quota of crickets to be added to each exposure test vessel on each day of the test were prepared in advance, by weighing selected crickets (between 1-2cm head to thorax length) using a flat pan balance. The total mass of crickets presented as food items an those crickets removed from each test vessel over the duration of the test, are shown in Table 8.4a, and summarised in Table 8.4b.

Figure 8.2 Mean body mass (g) over the duration of the scope for growth test with 95% confidence intervals (n=3 per exposure group).

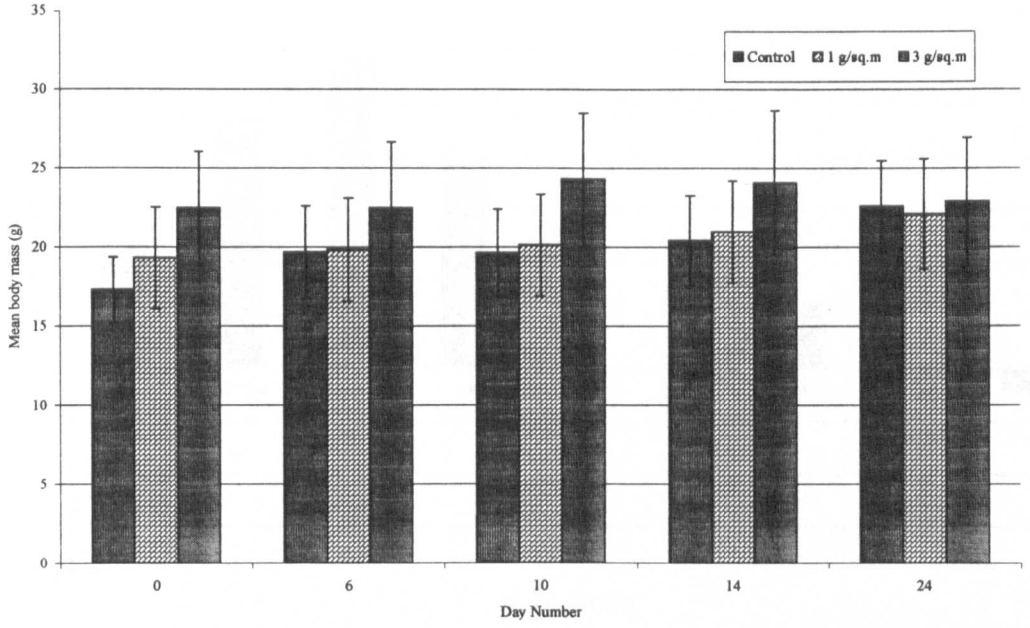


Figure 8.3 Body mass (g) gain or loss over the duration of the scope for growth test according to exposure group.

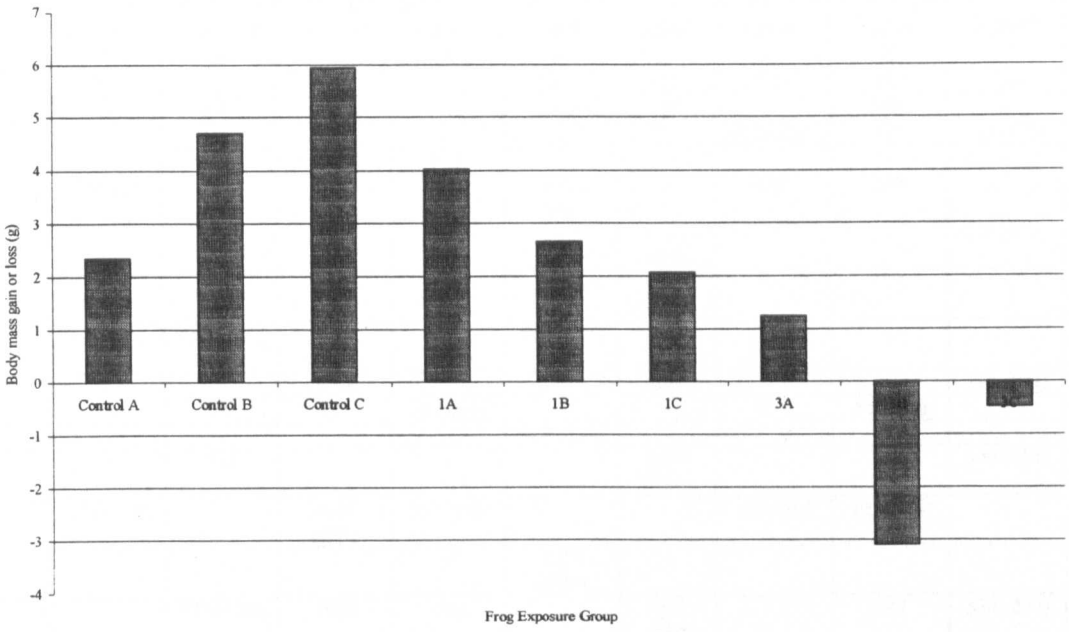


Table 8.4a Quotas of crickets (mg) presented to all frogs on each day of the test (A, B & C = frog replicate). Mass of crickets removed in brackets.

Day number	Individual exposure group (g/m^2 of NH_4NO_3 corrected for NO^-_3 N component)								
	Control A	Control B	Control C	1 g/m^2 A	1 g/m^2 B	1 g/m^2 C	3 g/m^2 A	3 g/m^2 B	3 g/m^2 C
1	683	759	633	486	609	858	1030	995	1104
3	766	897	728	750	863	507	-	601	-
4	-	-	-	-	-	727	621 (220)	530	513 (175)
5	-	-	1047	715	-	-	-	-	-
6	926 (165)	1188 (109)	-	-	1026 (219)	-	942	504	-
7	-	-	645	-	-	1081 (47)	-	-	618 (316)
8	-	-	-	1187 (329)	-	-	-	535	-
9	628 (44)	651 (237)	532	-	869 (237)	527	1019 (165)	-	-
10	-	-	1004	-	-	594	-	-	498 (243)
11	1055	596	977	708 (81)	-	-	807	538 (98)	-
12	-	-	-	-	919 (344)	-	-	-	-
13	-	989	-	-	-	862 (259)	-	-	500 (454)
14	574 (260)	-	1014 (271)	553 (110)	-	-	584 (46)	509 (148)	-
15	-	-	-	-	1028 (125)	-	-	-	-
16	-	973 (120)	1042	960	-	1162 (237)	-	507	534 (227)
17	974	-	-	-	568	-	574 (485)	-	-
18	-	-	575	681	-	541	-	-	-
19	-	993 (187)	-	-	510	908	-	538 (170)	543 (195)
20	1028 (258)	-	588	852	-	-	514 (444)	-	-
21	-	-	531	-	528	-	-	-	-
22	575	1072 (436)	532	973	-	987 (395)	-	558 (512)	513 (198)
23	-	-	597	-	-	-	491 (258)	-	-
24	523	-	-	611	552 (438)	-	-	-	-
25	531	673 (123)	538	-	-	495 (336)	-	506 (428)	521 (279)
26	-	-	539	583	-	-	493 (292)	-	-
27	521	-	660	-	498 (106)	-	-	-	-
28	-	503 (435)	504	499	-	519 (154)	-	631 (325)	509 (193)
29	509	-	-	-	-	-	533 (150)	-	-
30	-	528	498	518	548 (124)	-	-	543	-
31	527	-	502	-	-	534 (81)	-	628	721 (400)
32	-	508	508	-	-	-	533	-	-
33	540	-	-	586 (116)	565 (101)	-	-	556	-
34	-	585	496	532	-	526 (123)	-	-	736 (275)
35	-	520	-	-	574	-	568 (109)	-	-
36	528	542	502	1040	-	529	-	543	-
37	(174)	(166)	(126)	(219)	(208)	(169)	-	(335)	-
TOTAL	10888	11977	15192	12234	9657	11357	8709	9222	7310
Total (mg) crickets consumed	9822	10264	14795	11379	7755	9556	6003	7006	4655

Table 8.4b Cricket quota summary table for those crickets presented as food items over the duration of the test.

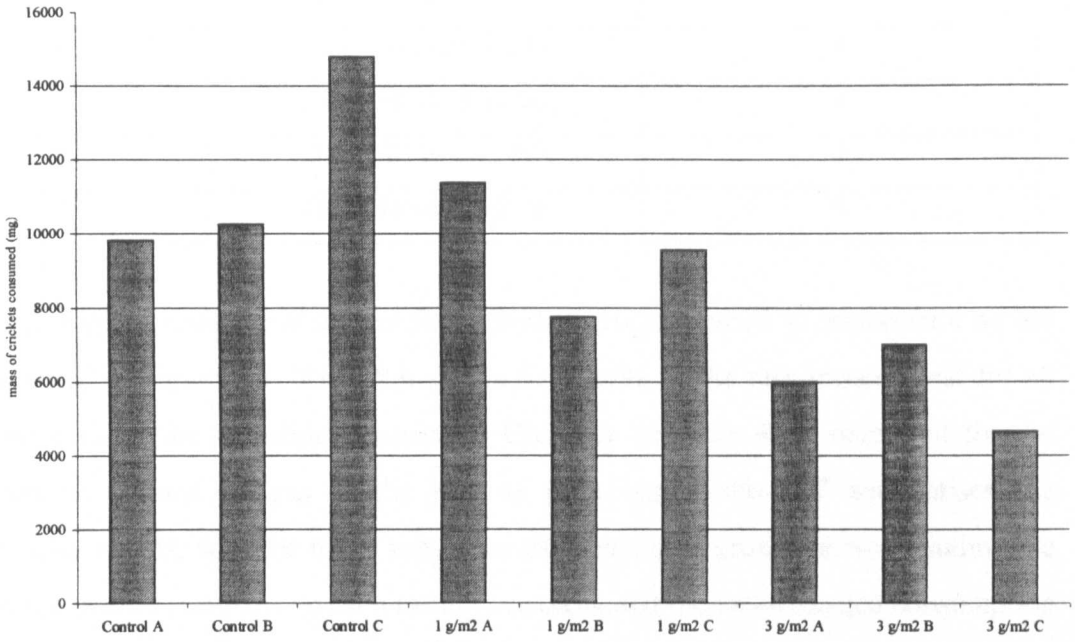
Parameter	Individual frog exposure group (g/m_2)								
	Control A	Control B	Contro l C	1 g/m^2 A	1 g/m^2 B	1 g/m^2 C	3 g/m^2 A	3 g/m^2 B	3 g/m^2 C
Total mass of crickets (mg)	10888	11977	15192	12234	9657	11357	8709	9222	7310
No. of feeding occasions	16	16	23	17	14	16	13	16	12
Average (mg)	680.5	745.6	660.5	719.6	690	709.8	669	576.4	609.2
Standard deviation	201.4	233.1	201.3	211.9	201.3	230.9	203.9	118.6	176.9
Standard error	50.4	56.3	52.0	51.4	53.8	57.7	56.5	29.7	51.1
95% Confidence interval	107.3	119.9	87.2	109.0	116.3	123.0	123.1	63.2	112.5
Cricket quota range (mg)	680.5 ± 107.3	745.6 ± 119.9	660.5 ± 87.2	719.6 ± 109	690 ± 116.3	709.8 ± 123	669 ± 123.1	576.4 ± 63.2	609.2 ± 112.5
Actual mass (mg) of crickets consumed by each frog.	9822	10264	14795	11379	7755	9556	6003	7006	4655

From Table 8.4 and Figure 8.4, it may be seen that the mass of crickets consumed over the duration of the test were greater in the control and 1 g/m^2 than at 3 g/m^2. Analysis of variance revealed that there was a significant difference between the mass of crickets consumed between groups, with the amount consumed by frogs at 3 g/m^2 being significantly ($f=6.21$; $p=0.035$; $df=8$) less than those crickets consumed by control animals. No differences were detected between frogs exposed at the 1g/m^2 exposure treatment and the other two groups.

8.3.4 Analysis of faecal pellets

All faecal pellets (where possible) were collected and frozen for bomb calorimetric analysis. Table 8.5 shows the number of faecal pellets collected from each test vessel throughout the test. Each pellet (or pooled sample of pellets if more than one pellet was collected from the same vessel on the same day) was oven dried at 60°C for 12 hours and then re-weighed to establish the dry mass of each sample. The exposure chambers at the two highest exposure concentrations were both badly soiled with faecal material, that made collection of faecal pellets impossible on two occasions; days 14 and 37. Due to time constraints, the complete set of faecal pellets were not combusted through the calorimeter, and subsequent values for their heats of combustion were not derived.

Figure 8.4 Mass (mg) of crickets consumed by all frogs at each exposure level. (A, B & C = replicates at each exposure concentration (NO^{-}_{3} N).



8.3.5 Adult frog respirometry

Adult common frogs were placed into the respirometer apparatus on 5 occasions over the 37 day exposure period. Oxygen consumption was determined using the techniques detailed in Section 8.2. Appendix 8.1 shows the Table of respirometer voltage readings. Testing was carried out between the 15 November 1996 and 21 December 1996. The possibility does exist that the individual frogs may have been affected by the season in which they were tested, with frogs BMR reducing as a result of environmental cues. The affects of season were minimised by housing the individuals under constant environmental conditions, but there still exists the risk of season affecting the BMR values. A single testing occasion was defined as the time taken to test all frogs from all exposure groups. Table 8.5 shows the time (days) taken to record voltage readings for all frogs on each of the testing occasions. The approximate time taken to test all frogs on each occasion was 4 days.

Table 8.5 Respirometer testing times for each exposure group of frogs.

Testing Occasion	Dates	Number of days to test all frogs on each test occasion
1	15.11.96 to 19.11.96	5
2	21.11.96 to 24.11.96	4
3	25.11.96 to 28.11.96	4
4	29.11.96 to 2.12.96	4
5	18.12.96 to 21.12.96	4

The mean oxygen consumption rates for individual frogs exposed to ammonium nitrate are presented in Figure 8.5. Table 8.6 shows the results of the respirometer test for all frogs on each of the 5 testing occasions. Chamber volumes were corrected for the displacement volume of gas at the end of each test. Table 8.7 summarises the respirometer results, with the mean values for each exposure group shown including the mean body masses recorded on each testing occasion, and the mean oxygen consumption per g or frog, and the associated standard error.

Table 8.6 Respirometry results for each frog on each test occasion showing values for ▲N in ml / O_2 (see Table 8.7 for body mass (g) summary).

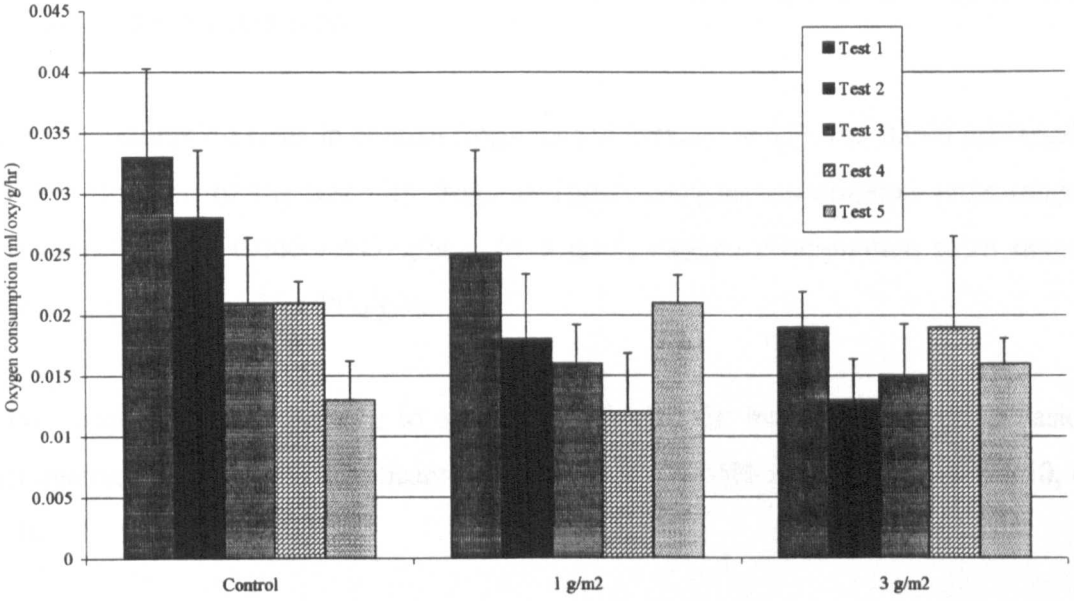
Test	Treatment	Chamber* volume (ml)	Voltage (v) difference	Derived water height (mm)	Water height in inches.	▲N Total (ml/O_2)	▲N ($ml/O_2/hr$) per g frog
1	1	997.5	2.37	23.7	0.96	0.68	0.021
1	1	969	4.12	41.9	1.70	1.18	0.031
1	1	970.6	6.17	63.2	2.57	1.78	0.046
1	2	922	3.81	38.7	1.57	1.03	0.022
1	2	999.5	5.01	51.2	2.08	1.48	0.046
1	2	983.5	1.51	14.7	0.60	0.42	0.019
1	3	981.6	3.20	32.3	1.31	0.92	0.026
1	3	948.2	2.77	27.9	1.13	0.76	0.018
1	3	881	3.60	36.5	1.48	0.93	0..017
2	1	1003.4	3.75	38.1	1.55	1.11	0.035
2	1	948.6	2.67	26.8	1.09	0.74	0.017
2	1	941.5	5.08	51.9	2.11	1.41	0.032
2	2	913.5	1.75	17.2	0.70	0.46	0.009
2	2	989.5	3.21	32.4	1.32	0.93	0.027
2	2	986.1	2.66	26.7	1.09	0.76	0.022
2	3	1002.7	2.29	22.9	0.93	0.66	0.021
2	3	943.3	1.64	16.1	0.65	0.44	0.010
2	3	859.3	2.83	28.5	1.16	0.71	0.012
3	1	998.6	1.84	18.2	0.74	0.53	0.016
3	1	958.2	2.01	20.0	0.81	0.55	0.014
3	1	938.6	5.01	51.2	2.08	1.39	0.031
3	2	911.8	2.05	20.4	0.83	0.54	0.011
3	2	981.7	2.78	28.0	1.14	0.79	0.022
3	2	987.1	2.23	22.2	0.90	0.64	0.018
3	3	988.4	1.12	10.7	0.43	0.31	0.009
3	3	921.6	4.09	41.6	1.69	1.11	0.023
3	3	845.1	3.13	31.6	1.28	0.77	0.012
4	1	993.6	2.84	28.6	1.16	0.82	0.024
4	1	947.3	2.82	28.4	1.15	0.78	0.018
4	1	931	3.75	38.1	1.55	1.03	0.022
4	2	905.6	1.48	14.4	0.59	0.38	0.007
4	2	988.3	2.65	26.6	1.08	0.76	0.022
4	2	961.6	1.24	11.9	0.49	0.33	0.008
4	3	994	4.00	40.7	1.65	1.17	0.035
4	3	937.6	3.46	35.0	1.42	0.95	0.021
4	3	829.5	2.58	25.9	1.05	0.62	0.009
5	1	974.1	2.61	26.2	1.06	0.74	0.020
5	1	922	2.26	22.6	0.92	0.60	0.013
5	1	911.2	1.79	17.7	0.72	0.47	0.009
5	2	884.8	3.69	37.4	1.52	0.96	0.017
5	2	973	2.76	27.8	1.13	0.78	0.021
5	2	963.2	3.50	35.5	1.44	0.99	0.025
5	3	866.9	3.23	32.6	1.33	0.82	0.014
5	3	912.6	3.45	34.9	1.42	0.92	0.019

Table 8.7 Respirometry results, showing mean body mass (g) per exposure group with mean oxygen consumption per hour per gramme of frog (n=3 per group). 1 standard error shown.

Test occasion	Exposure group	Mean body mass (g)	Oxygen consumption (ml / O_2 / g / hr)	Standard error (ml / O_2 / g / hr)
1	Control	18.26	0.033	0.007
1	1 g/m^2	19.32	0.025	0.009
1	3 g/m^2	22.47	0.019	0.003
2	Control	19.71	0.028	0.006
2	1 g/m^2	19.86	0.018	0.005
2	3 g/m^2	22.65	0.013	0.003
3	Control	19.64	0.021	0.005
3	1 g/m^2	20.14	0.016	0.003
3	3 g/m^2	24.32	0.015	0.004
4	Control	20.43	0.021	0.002
4	1 g/m^2	20.99	0.012	0.005
4	3 g/m^2	24.12	0.019	0.008
5	Control	22.58	0.013	0.003
5	1 g/m^2	22.13	0.021	0.002
5	3 g/m^2	27.19	0.016	0.049

On the first testing occasion the control frogs had higher oxygen consumption rates than those frogs exposed at 1 and 3 g/m^2. A one-way analysis of variance of oxygen consumption per g of frog against treatment was carried out on each testing occasion. There were no significant differences detected (at the 5% level) between the oxygen consumption rates of frogs between exposure groups, and between the amounts of energy expended during maintenance of a basal metabolic rate within the chambers. Energy values may be seen in Table 8.8.

Figure 8.5 Mean oxygen consumption rates for individual adult frogs exposed to ammonium nitrate fertiliser at control, 1 and 3g/m^2. Results are for measurements taken on five occasions over a 37day period. Time between tests is shown in Table 8.6. Standard errors are shown.



A One-way analysis of variance of oxygen consumption rates per g of frog, on each of the test occasions ($p<0.05\%$ level).

Test Occasion	Degrees of freedom	P value	Significance
1	8	0.456	Ns
2	8	0.214	Ns
3	8	0.672	Ns
4	8	0.418	Ns
5	7	0.234	Ns

Ns = no significance difference.

Oxygen consumption rates in control frogs ranged between 0.009 and 0.046 ml/O_2/g/hr over the duration of the test. In frogs at 1g/m^2, oxygen consumption rates ranged between 0.007 and 0.046 ml/O_2/g/hr. At 3 g/m^2, oxygen consumption rates ranged between 0.009 and 0.035 ml/O_2/g/hr.

A Two-way analysis of variance to assess the affect of the interaction of test occasion with treatment revealed no significant difference at the 0.05% level ($f=1.17$, $p=0.350$, $df = 44$).

The analysis of the data revealed no statistically significant differences between consumption rates of oxygen between frogs across all exposure groups. Test occasion was also found to have no significant affect ($f = 0.06$;, $p= 0.811$) on the consumption rates.

Table 8.8 Energy expenditure during maintenance of basal metabolic rates determined using the oxygen consumption rates per g or frog.

Test Occasion	Exposure Group	Oxygen consumption rate ml / O_2 / g / hr	Energy expenditure rate J / g / hr
1	1	0.021	0.441
	1	0.031	0.651
	1	0.046	0.966
	2	0.022	0.462
	2	0.046	0.966
	2	0.019	0.399
	3	0.026	0.546
	3	0.018	0.378
	3	0.017	0.357
2	1	0.035	0.735
	1	0.017	0.357
	1	0.032	0.672
	2	0.009	0.189
	2	0.027	0.567
	2	0.022	0.462
	3	0.021	0.441
	3	0.01	0.21
	3	0.012	0.252
3	1	0.016	0.336
	1	0.014	0.294
	1	0.031	0.651
	2	0.011	0.231
	2	0.022	0.462
	2	0.018	0.378
	3	0.009	0.189
	3	0.023	0.483
	3	0.012	0.252
4	1	0.024	0.504
	1	0.018	0.378
	1	0.022	0.462
	2	0.007	0.147
	2	0.022	0.462
	2	0.008	0.168
	3	0.035	0.735
	3	0.021	0.441
	3	0.009	0.189
5	1	0.02	0.42
	1	0.013	0.273
	1	0.009	0.189
	2	0.017	0.357
	2	0.021	0.441
	2	0.025	0.525
	3	0.014	0.294
	3	0.019	0.399

8.3.6 Faecal pellet production

The dry mass of all collected pellets was determined. The dry masses of all pellets collected during the 37 days of the test are tabulated in Table 8.9. Total pellets numbers produced and the mean masses per exposure group are displayed in Figures 8.6 and 8.7. It may be seen that frogs at the highest exposure concentration produced more pellets over the exposure period. An analysis of variance of dry faecal pellet mass against treatment reveals that a statistically significant difference exists between the mass of pellet at 3 g/m^2 and those pellets at $1g/m^2$ ($f=7.26$; $p=0.001$; $df=63$). From Table 8.9, the differences may be attributed to the effect of the higher number of lighter faecal pellets being produced by frog B compared to frogs A and C at $3g/sqm^2$. However, the mean mass of pellets produced by frogs B and C at 3 $g/sq.m^2$ is considerably less relative to the other treatments.

Table 8.9 Dry mass (mg) of faecal pellets collected from frog exposure tanks over the duration of the test.

Date	Frog exposure group								
	Control A	Control B	Control C	1 g/m$^+$ A	1 g/m$^+$ B	1 g/m$^+$ C	3 g/m$^+$ A	3 g/m$^+$ B	3 g/m$^+$ C
15.11.96	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0.0612	0	0.0594	0	0.0565	0.0215	0
19	0.0397	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0.0698	0	0
23	0.0500	0	0	0.0633	0	0	0	0.0361	0.0289
24	0	0	0	0	0	0.0488	0	0.0286	0
25	0	0.0325	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0.0226	0
27	0	0	0	0	0.0590	0	0	0	0
28	0.0257	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0.0403	0	0
30	0	0	0	0	0	0.0396	0.0436	0.0550	0
1.12.96	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0.0085
3	0.0465	0	0.0230	0	0	0.0488	0	0	0
4	0	0	0	0.0433	0	0	0	0	0.0094
5	0.0218	0.0258	0	0.094	0.0245	0.1161	0	0.0220	0
6	0	0	0	0	0	0	0	0	0
7	0.0589	0	0	0	0	0.0528	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0.1249	0	0.0143	0	0	0	0.0281	0	0.0294
10	0	0.0367	0	0	0.0231	0.0192	0	0	0
11	0	0	0	0	0	0	0	0.0152	0
12	0	0	0	0	0	0	0	0	0
13	0	0.0349	0	0	0	0	0.0262	0	0
14	0	0	0	0	0	0	0	0.0456	0.0091
15	0	0	0	0	0	0	0	0.0260	0.0040
16	0	0.0331	0.0276	0	0.0294	0.0748	0.0425	0	0.0138
17	0.0615	0	0	0.0644	0.0321	0.0525	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0.0298	0.0263	0	0	0	0	0.0208	0.0153
20	0	0	0	0	0	0	0	0.0147	0.0142
21	0	0	0	0	0	0	0	0	0
TOTAL	0.429	0.1928	0.1524	0.265	0.2275	0.4038	0.307	0.3081	0.1326
No. of pellets	8	6	5	5	6	8	8	13	11
Mean mass (mg)	0.054	0.032	0.031	0.053	0.038	0.05	0.038	0.024	0.012

An analysis of variance shows that a significance difference was detected between the number of pellets produced ($f = 5.12$; $p = 0.050$; $df = 8$). Figures 8.8 – 8.10 show the cumulative production rates of faecal pellets over the duration of the tests at each of the exposure concentrations. The rate of production was assessed by linear regression of the cumulative production rates at each exposure concentration. In each instance, the graph displays the linear regression lines, showing the R^2 correlation coefficients for each trendline. Table 8.10 shows the regression coefficient values obtained on each plot.

Table 8.10 Correlation coefficients for faecal pellet production rates during exposure to ammonium nitrate

Adult Frog Exposure Group		Correlation coefficient (R^2 value)
Control	Rep A	0.9683
	B	0.9128
	C	0.9282
1 g/m^2	A	0.9738
	B	0.9542
	C	0.8315
3 g/m^2	A	0.9712
	B	0.9785
	C	0.9686

Comparing the gradients of the regression lines obtained in each case assessed the rate of pellet production between exposure groups. The correlation coefficients obtained for these lines are very similar (R^2 values) with the exception of replicate C at 1g/m^2, which had a lower faecal pellet production rate. No difference in rates of faecal pellet production over the duration of the exposure period between exposure groups was detected. This suggests that exposure concentration had little of no impact on the rates of food assimilation, therefore not affecting the faecal pellet production.

Figure 8.6 Mean dry faecal pellet mass per frog at each exposure group over the duration of the test.

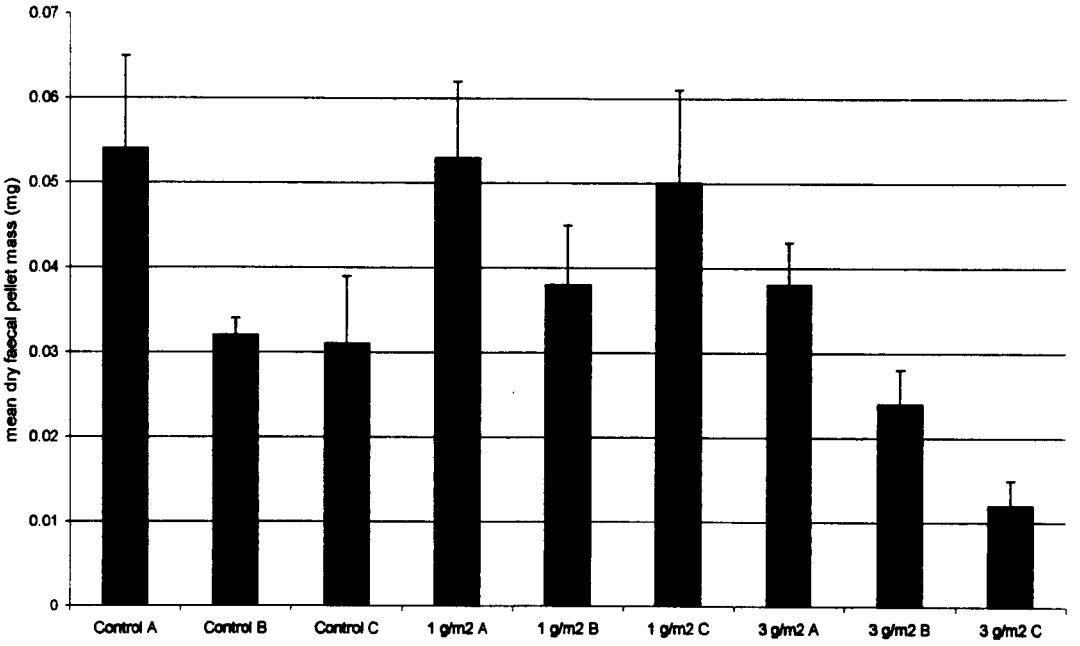


Figure 8.7 Total number of adult frog faecal pellets during the 37 day exposure period of the food conversion efficiency assay.

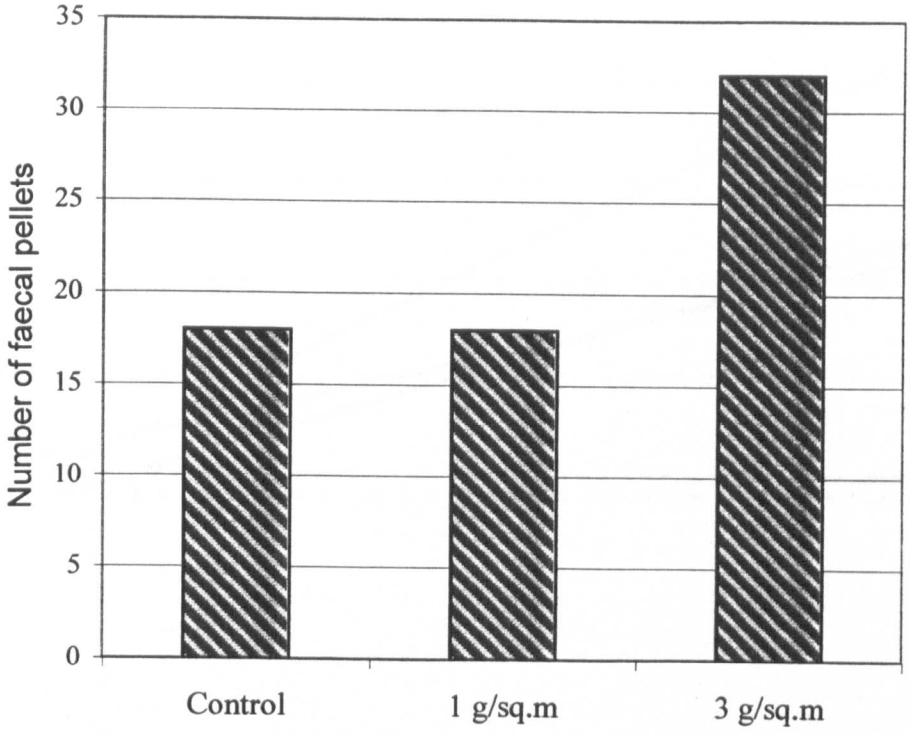


Figure 8.8 Cumulative faecal pellet production rate in the three replicate control frogs over the duration of the exposure period.

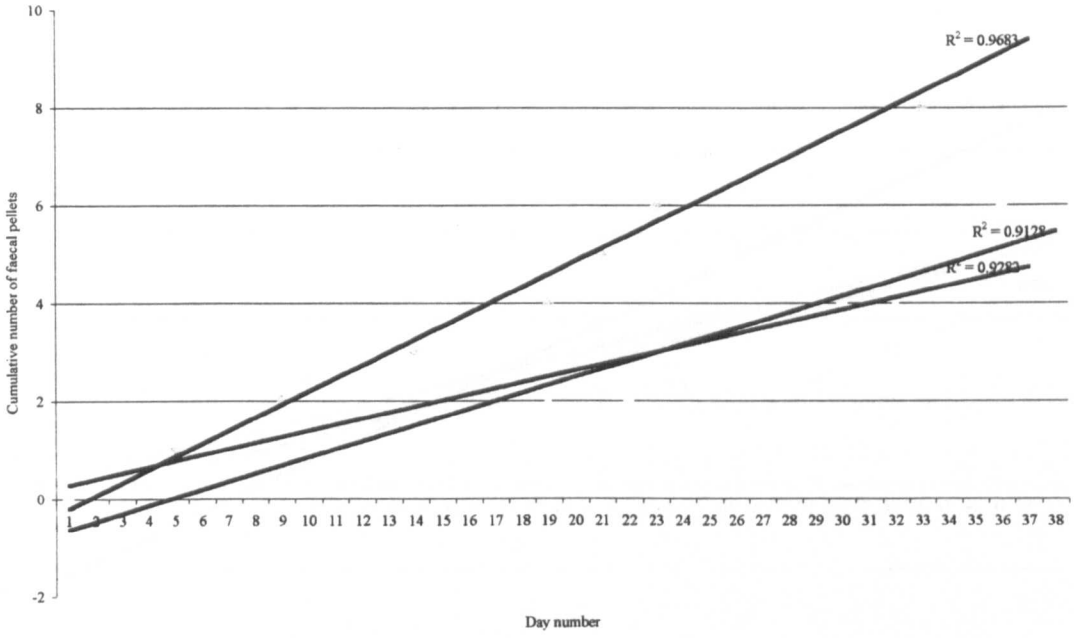


Figure 8.9 Cumulative faecal pellet production rate in frogs exposed at 1 g/m^2 over the duration of the exposure period.

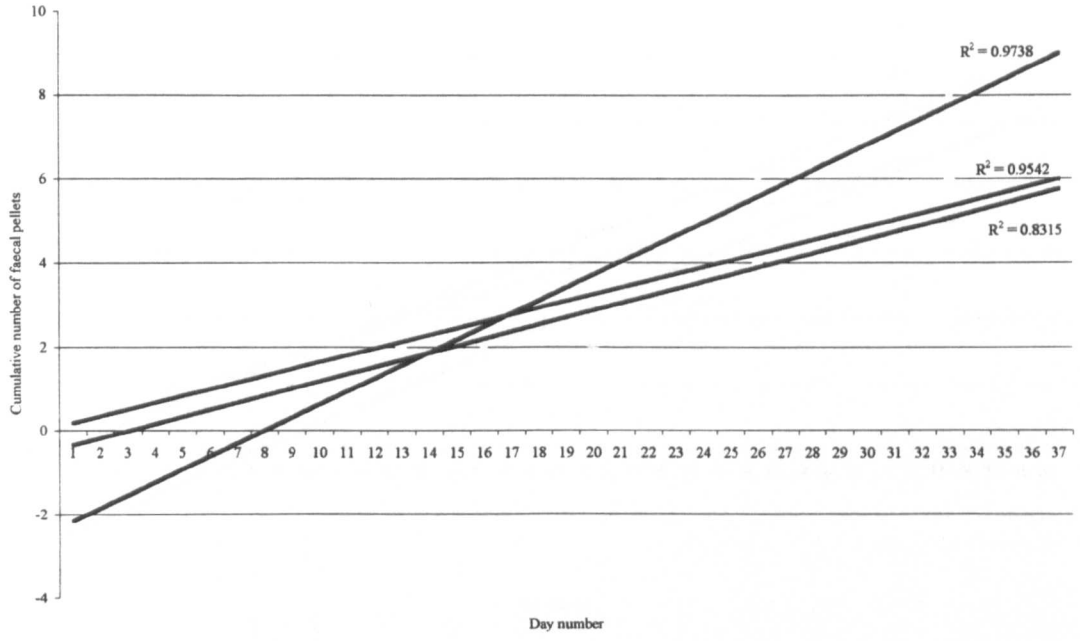
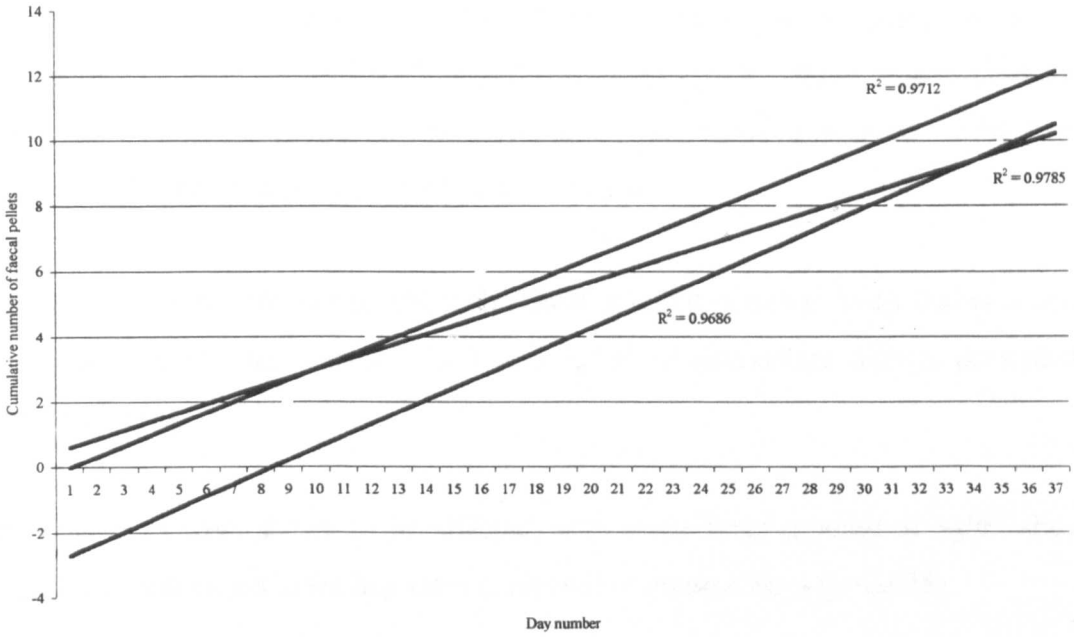


Figure 8.10 Cumulative faecal pellet production in frogs exposed to ammonium nitrate fertiliser at 3 g/m^2 over the duration of the exposure period.



8.4 DISCUSSION

This chapter has followed the development and application of a respirometer suitable for use with adult common frogs. The BMR (basal metabolic rate) for adult common frogs was found to range between 0.007 and 0.046 ml /O_2/g /hr. These values did compare favourably to values cited from the literature. It appeared from these results that the design of the test system was robust and functional.

Ammonium nitrate did not to affect the BMR of adult common frogs during a chronic exposure period when exposed to 1 and 3g/m^2 of ammonium nitrate, presented on uniform substrates.

Feeding rates were shown to be affected, with those frogs exposed at 3g/m^2 showing significant reductions in feeding rates compared to control frogs ($p=0.035$).

Body mass was significantly reduced over the study period, with one out of three individuals dying at the highest exposure concentration. This reduction in body mass may be related to feeding rate, where at the highest concentration, feeding rates (mass of crickets consumed) were significantly less than controls. The numbers of faecal pellets produced was also shown to increase significantly with exposure concentration. However, the faecal pellet dry masses over the duration of the test were significantly less at higher exposure concentrations than in control groups. Although I was unable to utilise the bomb calorimeter completely, the effects of exposure to up to 3 g/m^2 ammonium nitrate (as NO^-_3 N) on individual adult frog fitness has the potential to reduce productivity (body mass). Basal metabolic rates (BMR) appear not to be affected. Faecal pellet production increased significantly, with the rate of production remaining unaffected. The mean weight of faecal pellets was significantly reduced with increasing concentration. Feedings rates were significantly reduced with increased concentration. Overall, it can be said that an increase in ammonium nitrate concentration has been shown to affect individual fitness of adult common frogs, by affecting productivity and feeding behaviour.

CHAPTER NINE

DISCUSSION

CHAPTER NINE
DISCUSSION

The aim of this study was to investigate sub-lethal effects of ammonium nitrate fertiliser on each developmental stage of an amphibian, the common frog *Rana temporaria*. The detrimental effects of pesticides and industrial chemicals on amphibians have been well documented (Chapter 1). However, the effect of common fertilisers on common frogs within suitable habitats has not been extensively researched. This study aimed to develop an insight into these effects and attempt to quantify those effects by means laboratory and field experiment.

This research has shown that residual nitrate concentrations in many standing water bodies resulting from leaching from agricultural land in modern intensive farming areas are high. Vernal applications of ammonium nitrate coincident with the annual migration result in high concentrations of nitrate in breeding sites of common frogs, introducing the potential to modify indigenous populations of common frogs.

The results have demonstrated direct and indirect toxicity of ammonium nitrate on the common frog. The concentration having a range of effects dependent on the developmental stage. Both acute and chronic exposure to sub-lethal concentrations of ammonium nitrate led to sub-lethal effects that were quantified during tests with captive populations of common frogs.

The specific objectives of the study were to:

- To establish residual concentrations of nitrate in standing water bodies within agricultural environments, where common frogs were likely to reproduce.

- To investigate the impact of ammonium nitrate on the growth and development of common frogs spawn and larvae.

- To assess the effect of ammonium nitrate on the behaviour of adult common frogs.

Residual concentrations of nitrate in pond water during the breeding season of the common frog recorded over two years of monitoring were between 0 (<3mg/l NO^-_3 N) and 139 mg/l NO^-_3 N. The majority of ponds sampled (58 %) were supplied with water by either rainfall or run-off alone, from adjacent agricultural land. Ponds fed by field drains, (25 % of the sample) had the highest recorded concentrations of nitrate. The highest residual concentration of nitrate recorded for a pond where common frogs were found was 96 mg/l NO^-_3 N. A field drain also fed this pond. The majority of amphibians (68 %) were found in sites fed by rainfall and runoff, and not in those ponds fed by land drains. The nitrate monitoring programme established that exposure concentrations of at least 100mg/l NO^-_3 N, in the laboratory were representative of the concentrations reported in field ponds, where common frogs were found. It is postulated that common frog populations will tolerate a wide range of residual nitrate concentrations in the field. The greatest number of common frog spawn clumps (n = 85) was found at a site where residual nitrate concentrations were below the levels of accurate quantification for the entire period of monitoring. This in part, suggests that for these sites, the number of spawn clumps and therefore the size of the common frog breeding population appears to be high. No significant correlation was found between the way in which site water was supplied and the diversity of amphibians present at a site. Where a correlation was hinted, it may suggest that some other factor such as water quality, or the presence of predators such as aquatic invertebrate larvae or even fish present in the ponds, may well have affected the assemblages of amphibians present. Even human intervention in those rural sites near to residential may have succumbed to the wondering interests of gardeners, removing spawn clumps ultimately affecting the recruitment into subsequent populations. It appeared that the common factor in determining the presence or absence of frogs, were the levels of residual nitrate for those ponds over the monitoring period. Residual nitrate concentrations were high during the winter months with high concentrations present into the vernal period of the following year. Concentrations of nitrate reduced in the months approaching summer (May and June). During the summer months, coincident with periods of low rainfall, levels of nitrate dropped. This also coincided with increased productivity in pond sites.

Common frog spawn was collected from sites where low residual nitrate concentrations were recorded during the nitrate sampling programme, and exposed to a pulse of ammonium nitrate in solution, and chronic exposure to a range of ammonium nitrate concentrations. Embryo survival was affected by nitrate concentration with 87% (n=92) of a test population hatching, compared to 63% (n=86) hatching success in individuals exposed at 80 mg/l NO^-_3 N.

Where embryos were exposed to moderate levels (up to 40mg/l NO^-_3 N) of ammonium nitrate in solution, jelly sac diameters were significantly increased, affecting subsequent survival of developing embryos.

Krogh & Ussing (1938) discovered at certain times during the embryological development within the egg sac, the jelly sac shows a plasticity in permeability, which can dramatically reduce survival when embryos are exposed to concentrated solutions (10%) of NaCl. Following fertilisation, frog spawn will tolerate a certain amount of swelling. This occurs at a late stage in the development of the blastula. Once the gastrula begins to elongate, the changes in embryo volume are hard to measure but were found not to increase significantly. They found that if they increased the ionic concentration of the surrounding media by 10% using NaCl, blastula development was delayed and after 13 hours, those embryos exposed to 10% NaCl were dead. This suggests that the increased permeability of these embryos to the concentrated media was the cause of the increased mortality. To substantiate that the blastula stage appeared to be the most sensitive, they exposed developing embryos to untreated water until post blastula stage, and then once gastrulation had started, exposed individuals to the concentrated media. Normal development followed. With these results in mind, it was suggested that jelly sac permeability be significantly reduced following the onset of gastrulation. In the current study, increased mortality was observed with increasing concentration of nitrate in the surrounding media. It is argued that the osmotic potential between the internal environment of the developing embryo's and the external media was so great that at post gastrulation stages, the integrity of the jelly sac was compromised and subsequent mortality resulted. Where developing embryos were exposed at between

stages 9 and 13, mortality followed quickly and appears to corroborate the earlier findings of Krogh & Ussing (1904).

Work on the effect of ammonium nitrate on the larvae of the common frog indicated that ammonium nitrate was moderately toxic to developing larvae at stage 23 (Gosner 1960) of development, with a 48hour EC_{50} and 96 hour LC_{50} values of 398.7 and 781.1 mg/l $NO^{-}_{3}N$. These are the first recorded data of this kind for larvae of the common frog. In *Rana pipiens* and *Rana clamitans*, 96 hour LC_{50} values have been obtained of 22.6 and 32.4 mg/l NO^{-}_{3} N respectively, Hecnar (1995). The values obtained for EC_{50} for *Rana temporaria* in this study are an order of magnitude greater than those found by Hecnar.

The variation in the LC_{50} values may be due in part to climatic differences that exist between continents. The biome affect may well cause similar species to respond in different ways. This may be due to genetic conditioning because of evolution, a speciation affect, or may be because of the differences in the ways in which man has influenced particular habitats. It is notable that the relative sizes of suitable ecosystems in Canada are substantially greater than those found in the United Kingdom. The influence of man on the environment and the resultant potential resistance to cultural additions such as ammonium nitrate on resident amphibian populations may also be considerably reduced when compared to British indigenous populations, where agricultural development has been more intensive for a longer period than in Canada. This may have led to the reduced nitrate resistance and lower effect concentrations revealed by Hecnar.

Hecnar (1995) did suggest that his findings were possibly due to amphibians being differentially exposed to nitrate before collection, suggesting a 'patchwork' exposure compared to the more intensive nature of agriculture in the United Kingdom. This suggests that the above argument concerning the geographical impact of man in Canada in the testing area led to variations in the resistance of individuals to cultural additions. It also suggests that possibly the effects of genetic drift between neighbouring amphibian populations within large biomes much less evident, leading to differential resistance.

It has been shown that amphibians do have the ability to develop a chemical resistance over time. Organophosphorous pesticides that act as cholinesterase inhibitors in target invertebrate and vertebrate species, are able to accumulate or sequester pesticide into lipid tissue and the liver or certain anuran species such as the bull frog (*Rana catesbiana*) Hall & Kolbe (1980). In the current study, the study sites monitored over the two-year period, it was with confidence that the animals used in this study were not differentially exposed to nitrate. It is interesting to note that the common toad had a 96 hr LC_{50} of value 1637 mg/l $NO^{-}_{3}N$ using ammonium nitrate (Xu & Oldham (1997). The common frog shows double the sensitivity to ammonium nitrate than the common toad. When these values are compared with Hecnars (1995) findings, the values are insignificant when compared to the acute toxic concentrations recorded for *R. clamitans* and *R. pipiens*, revealing that there appears to be inter species differences in sensitivity to the acute toxic effects of ammonium nitrate to amphibian larvae.

Continuous exposure of larvae to the range of nitrate concentrations obtained in the field showed that there was a high survival until stage 40-42 (Gosner 1960), thereafter, during metamorphosis, high levels of mortality were recorded at the higher exposure groups. This was investigated under chronic exposure conditions, where the test system was designed to maintain nitrate concentrations for the duration of the test under continuous renewal conditions. Criteria were laid down as to the design of the test system and were achieved. The test system was successful in maintaining a large number of larvae over a chronic exposure period.

From the results shown in Chapter 6, it was indicated that the most sensitive phase when mortality occurred in the development of the larvae was between front limb emergence (Stage 40) and froglet emergence (stage 44, (Gosner 1960)), where high rates of larval mortality occurred. Until the first larvae were observed with front limbs, larval survival was more than 90 % in all exposure concentrations. Subsequently, mortality rates increased across all nitrate treatments, with mortalities in the control group also increasingly slightly, but not significantly compared to the treated groups. Larval body mass and length increased significantly ($p<0.05$) at greater rates in controls relative to the treated larvae, with body lengths negatively correlated with concentration ($P<0.05$). Mortality rates at the higher concentrations of ammonium nitrate were high. Baker &

Waights (1993) found that common frog tadpoles suffered relatively high mortality when exposed to concentrations of sodium nitrate. However, Baker & Waights' used concentrations of sodium nitrate prepared using distilled water. It was found during the course of their study, that the use of distilled water, rather than artificial pond water, led to reduced productivity and lower feeding rates in common frog tadpoles.

The initial phases or exponential phases of growth and development are the most important for species existing within ephemeral habitats. Those individuals that attain a larger size at an earlier stage in a population's existence carry an advantage into subsequent phases of the life cycle and will have the ability to out compete smaller individuals for resources within a chosen habitat Goater (1993). It has been reported that the common newt *Triturus vulgaris* and common toad *Bufo bufo* are both affected at a sub-lethal level by ammonium nitrate (Watt & Oldham, 1995, Xu & Oldham 1997 respectively). Feeding behaviour, body size and survival rates were all affected in both cases. With newts exposed at high concentrations of ammonium nitrate (200 & 500 mg/L) being significantly smaller than controls at metamorphosis. Larvae at 100mg/L had significantly higher feeding rates than controls, although not reflected in their size at metamorphosis. Also, there were indirect affects on newt prey items, with Daphnia exposed at 200 & 500mg/l more likely to be found at the top of the water column. In toads, decreases in toad activity levels were also observed, with reductions in the feeding activity and changes in swimming behaviour was also noted at the higher test concentrations.

In the present study, feeding behaviour was not assessed directly but only through cursory inspection over the duration of the chronic study.

In common toads (Xu & Oldham (1997) deformities were observed in larvae exposed up to 50mg/l NO^{-}_{3} N. This study revealed differences in the frequency and severity of abnormality reported in common frog larvae, with some affected larvae surviving through to the final stages of metamorphosis, at which point mortality rates were seen to increase significantly. The type of deformities observed in this study included gut malformations (malrotations), kinked tails, cranio-facial

dysplasia, dermal blisters oedema, and were similar in nature to those abnormalities observed in common toads (Xu & Oldham 1997).

Xenopus laevis embryos have been used to assess the teratogenic effects of complex mixtures of chemicals (Dumont 1982). Effects are detected at a sub-lethal level, through the assessment of malformations in the developing embryos. The assay known as the FETAX (**F**rog **E**mbryo **T**eratogenesis **A**ssay: **X**enopus) assay has revealed that type and degree of malformation observed during development, can be used to discriminate between particular chemicals. This has resulted from extensive evaluations of the assay between 1983-1991.

The FETAX method of analysis was developed by Dumont, Schultz, Buchanan & Kao (1983, and was designed to complement existing fish and invertebrate toxicity tests. During the development of the assay, the FETAX assays sensitivity to metals was recognised, with the assay now being an accepted ASTM (American Society of Testing Methods) testing standard since 1991, Dawson, Fort, Newell & Bantle (1989), Dawson & Wilke (1991).

Organism performance is at the heart of ecological and evolutionary processes, with any variation in individual performance becoming a focus of physiological ecology. The results of chapter 7 reveal that exposure to ammonium nitrate fertiliser at between1 and 3 g/m^2 in the laboratory had the potential to modify activity levels of captive common frog adults, and alter choice / preference behaviour. Adult frogs showed a preference for areas not exposed to ammonium nitrate fertiliser. If this applies during migration to breeding sites lowering the chances of reproductive success may reduce the population's fitness. With fertiliser application times being coincident with the common frog migratory season, it is suggested that there is a high risk to adult common frogs of a detrimental effect occurring during the migration process, for those populations located within arable agricultural areas. It is already known that adult common frogs are particularly sensitive to ammonium nitrate (Oldham *et al.* 1997) in the laboratory with an EC_{50} of 3.6 g/m^2.

The sensitivity of frogs to ammonium nitrate was slightly reduced when the experiment was repeated under field conditions, with an EC_{50} of 6.9 g/m^2. The results obtained in the laboratory are indicative of a worst case scenario, and are not subject to the surface variation that occurs with field soil and the impact of uncontrollable soil moisture levels.

To establish the impact of ammonium nitrate on common frog productivity, it was proposed to develop and apply a 'Scope for Growth' assay. By combining the impact of exposure on respiration and assimilation efficiency, it would be possible to predict the potential effects on individual adult frogs in the field. To achieve this, individual common frogs were exposed under controlled conditions, with measurements made on their basal metabolic rates, ie. Their resting rates of oxygen consumption and their feeding rates both under conditions of ammonium nitrate stress. It was appreciated that in the field, the calorific value of different prey items may be different to those proposed by feeding on a diet of house crickets. In addition, the impact of fluctuations in environmental temperature and conditions may also influence respiratory ability and feeding efficiency.

The respirometer designed by Gilson (1963) was used for the development of the frog respirometer. Gilson relied on maintaining a constant volume within a test chamber, by manipulating the test chamber volume. Periodically, the volume within a chamber would be corrected for by adding a gas volume to a chamber using a manometer screw in Gilsons Respirometer design. Where s low oxygen consumption was expected, it would be possible to record pressure change over a long period without interfering with the apparatus and disturbing the animal under test. Hence, a pressure transducer was used to monitor pressure change between two interconnected chambers. Given the sensitivity of the transducer, a slight pressure change due to the consumption of oxygen by the frog resulted in a voltage change across the diaphragm in the transducer. This was then directly related to oxygen consumption mathematically. Ammonium nitrate appeared not to affect the consumption rates of oxygen over the duration of testing, but the assay did reveal that productivity within frogs was severely affected. The net gain or loss of body mass during the study revealed that those individuals exposed at $3g/m^2$ exhibited significant body mass loss over the 37-day duration of the study. Little or no differences were seen in the body masses recorded in controls and individuals exposed at 1 g/m^2.

Such losses may suggest that for individuals exposed to ammonium nitrate approaching the recognised LC_{50} concentration, would result in a loss of productivity, ultimately affecting reproductive ability. If there is an additional effect on the feeding ability of the frog, then the effect is compounded. It was shown that over the duration of chronic feeding experiment, common frogs exposed at 3 g/m^2 handled fewer prey items relative to control animals. Over the duration of the study, the total mass of crickets consumed by each frog from each treatment group revealed significantly reduced total cricket mass consumed by individuals at the highest concentration. A significant difference was detected between the mass of faecal pellets produced at 3 g/m^2 and the lower exposure concentration of 1 g/m^2. This can be related to individuals at the higher concentration consuming fewer prey items over the duration of the test.

The respirometry results show additional problems with the use of captive frogs for the study. From Figure 8.6, the test design may be seen to be functioning in the control frogs. Comparison between the five individual testing occasions in the controls reveals a net reduction in the volumes of oxygen consumed. Over the first three occasions, this trend appears to follow for the remaining concentrations, with reductions at 1g/m and 3 g/m^2. At 3 g/m^2, the trend was less apparent compared to 1 g/m^2. However, frogs exposed at 3g/m2 relative to the other treatments produced a greater number of faecal pellets. This suggests that the content of faecal pellets at the higher exposure concentrations had a higher liquid content or was much smaller. However there were significant differences between the treatment groups possibly as a function of the small size of the treatment groups. The volumes of oxygen consumed do however appear to be similar with the range of consumption rates discussed earlier. The mean values between the three frogs at each exposure concentration in the control reduce over the five occasions. The animals appeared to be more acclimated to the conditions.

Some of the findings do highlight the plight of the common frog during its life history when presented with an agricultural landscape in which to develop and grow. Not only are there risks from predators within habitats, there are invisible risks from chemicals in the environment, which at certain concentrations are unavoidable. It has to be accepted that it is impossible to predict with complete confidence how a particular chemical will affect a particular species. The combination of environmental effects and the interaction

of a whole gambit of other factors, such as synergism, magnification, antagonism between different chemicals all contribute to the overall picture of a particular pollutant within a system. The distribution of a particular chemical in the environment will dictate which compartments within a particular habitat will be affected. Given the nature of the common frogs' life cycle, the affected habitats are wide and varied. Affects have been demonstrated in the aquatic and terrestrial phases of the frog's life cycle, at a crucial time during the development of a population. The point at which individuals reproduce is the most sensitive to the affects of perturbations. If the equilibrium is disturbed to such a point that equilibrium is not re-established, then populations will fail. In this case, if frogs are prevented from reaching a natal pond to reproduce, then the success of that population of recruiting into subsequent populations is diminished. Affects that manifest following successful fertilisation, have been shown to lead to reduced survival, which again has implications into the fitness of affected populations, due to the reduced chance of reproductive success due to reduced numbers of individuals surviving the aquatic phase of the life cycle. Although limited affects on growths rates were observed during this current study, it was observed that the most sensitive time in the development appeared to be the larval period as individuals start to metamorphose from free swimming larvae into froglets. The highest mortality was observed in individuals exposed during the point of first limb emergence and final emergence from the aquatic habitat. It would be useful to establish the impact of ammonium nitrate on the tail resorption processes that are in evidence during the essential moments of metamorphosis. This could provide evidence that ammonium nitrate exposure interferes with endocrine function, with a slowing of the tail resorption rate indicating an inhibition of endocrine function and an increased tail resorption rate indicating a stimulation of endocrine function. Given that to set up such a test would be simper than a life cycle test or could be combined into a life cycle test, the mode of action of ammonium nitrate on the development of the common frog could be established.

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APPENDICIES

APPENDIX 1

Measured concentrations of residual nitrate (mg/l NO_3^--N) recorded in the second flow-through test measured using the Reflectoquant portable nitrate meter 'RQ-Flex'. Values in brackets are daily measured concentrations expressed as a percentage of the values recorded on Day 0 for each fertiliser concentration.

Day Number	Measured concentrations mg/l NO_3^-N				Measured concentration as a % of the nominal concentrations with % of Day 0 values in brackets.			
	Control	25	50	100	Control	25	50	100
0	-	19	61	109	-	76 (-)	122 (-)	109 (-)
1	-	18	65	108	-	72 (95)	130 (107)	108 (99)
2	-	16	66	104	-	64 (84)	132 (108)	104 (95)
3	-	19	64	109	-	76 (100)	128 (105)	109 (100)
4	-	26	63	125	-	104 (137)	126 (103)	125 (115)
5	-	29	54	121	-	116 (153)	108 (89)	121 (111)
7	-	34	50	119	-	136 (179)	100 (100)	119 (109)
10	-	37	39	103	-	148 (195)	78 (64)	103 (94)
18	-	31	49	104	-	124 (163)	98 (80)	104 (95)
26	-	34	56	110	-	136 (179)	112 (92)	110 (101)
28	-	28	52	114	-	112 (147)	104 (85)	114 (105)
35	-	26	59	126	-	104 (137)	118 (97)	126 (116)
43	4	21	58	117	n/a	84 (111)	116 (95)	117 (107)
45	-	18	74	139	-	72 (95)	148 (121)	139 (128)
51	-	19	54	101	-	76 (100)	108 (89)	101 (93)
59	3	24	53	98	n/a	96 (126)	106 (87)	98 (90)
68	-	21	70	84	-	84 (111)	140 (115)	84 (77)
76	-	23	62	82	-	92 (121)	124 (102)	82 (75)
79	-	33	85	81	-	132 (174)	170 (139)	81 (74)
82	6	31	81	65	n/a	124 (163)	162 (133)	65 (60)
86	-	27	65	89	-	108 (142)	114 (107)	89 (82)
90	-	17	57	95	-	68 (89)	114 (93)	95 (87)
93	-	21	58	97	-	84 (111)	136 (95)	97 (89)
96	-	26	56	99	-	104 (137)	112 (92)	99 (91)

Average	**0.542**	**24.9**	**60.5**	**104.1**

APPENDIX 2

SITE DESCRIPTIONS IN 1994 (Including Plates).

1. **Wood Pond.** Grid ref. SK 665 053. (Plate One)

The Wood Pond was on land belonging to the Quorn Hunt in Leicestershire. Access permission was obtained from a local farmer who supervised the land for the hunt; occasionally the field was used as a horse pasture. The pond is located at the margin of an unimproved pasture bordering onto mixed woodland, split by a hawthorn hedge. The pond was approximately 5 meters long by 3 meters wide, with water depth of 2 meters at the centre. On the woodland side of the pond, was a gentle slope into the water, with emergent vegetation. On the pasture side, the pond sides were sheer, with little or no emergent vegetation. The pond had a surface area of approximately 15 sq.m. Access was gained via an access road (usually impassable due to flooding) or via a path through a small wood approximately a mile from the laboratories at Scraptoft. This site contained one sampling point located on the south bank of the pond *Rana temporaria* and *Triturus vulgaris* bred at this site.

2. **Fish pond** Grid reference: SK 661051 (Plate Two)

Fish Pond is located on a mixed arable sheep farm, approximately a mile from the laboratories on Covert Lane, Scraptoft. The pond lies at the margin of a winter wheat crop and an over winter sheep pasture. The pond collected water draining mainly from a grassland pasture via land drains, with the inlets and outlets clearly visible. Three points were sampled: inlet, outlet and main body of pond. The pond contained common carp and golden Orf. In spite of this, *Triturus vulgaris* was found in this pond.

3. **Nature Reserve Pond.** Grid reference SK 661053 (Plate Three)

Nature Reserve Pond is located on Mr. Renner's Farm on Covert Lane, Scraptoft. The pond was located at the top of a slope at the margin between an unimproved pasture and winter wheat crops on two sides, divided by a Hawthorn hedge. The pond has amenity value as an area of conservation on this farm, with the land (1/4 acre) fenced in and managed. Logs and wild plants a deciduous trees have been planted planting to give the area a wild feel. The pond is shallow, approximately 4

feet deep, with gentle slopes on all side. One sampling points; main-body of pond. *Rana temporaria* and *Triturus vulgaris* bred at this site.

4 & 5 **Keyham Pond and Ditch** Grid reference SK 663058 (Plate Four)

These two sites were located a mile from the laboratories, approximately ten minutes walk from the Wood Pond site. The ditch was located along the margin of winter wheat field. The ditch was fed by surface runoff from this field. Keyham Ditch fed Keyham Pond. The ditch was considered as a separate site due to its size and the presence of large areas of standing water. The pond was found at the junction of three fields, receiving drainage from two winter wheat fields and a maize field. This was a shallow pond (approx. 1 meter deep) with a surface area of 9 meters. The ditch was shallow, with a depth of less than 0.5 meters, and a width of approx. 1 meter, which ran along the entire length of a winter wheat field (200 meters). One sampling point in both ditch and pond, as central as was possible given the impassable nature of the site. *Rana temporaria* and *Triturus vulgaris* were found in the pond, with *Rana temporaria* in the ditch.

6. **Far Corner Field Pond** Grid reference SK 676148 (Plate's Five, Six & Seven)

Far Corner Pond situated towards one end of a winter wheat field completely surrounded Winter Wheat, that belonged to the Brooksby Agricultural College, Brooksby, Leicestershire. Immediately adjacent to the pond was a small stand of willow trees and some shrub foliage including blackthorn and Cows Parsley. An area of scrub, approximately 25 square meters was located on one side of the pond where no trees were present. The pond was fed with water from field drains with both the inlet and outlet with outlet sump, easily visible. Three sampling points were identified; field drain **inlet, outlet** and a central location between the inlet and outlet to sample the main body of the pond, known as the **central** point. Maximum water depth was approximately 1.6 meters. The pond surface area was approximately 90 square meters. Amphibians were breeding at this site in all years of the study, with spawn and eggs being found of *Rana temporaria, Triturus vulgaris* and *T cristatus.*

7. **Spinney field pond** Grid reference SK 682 153 (Plate Eight)

Spinney pond was located between a mixed broadleaf woodland, a rape crop field and a maize crop field. Immediately adjacent and surrounding the pond, were stands of Willow and Oak trees with Hawthorn and Dogthorn bushes in evidence. Between the rape crop, field and maize crop field was a semi-continuous Hawthorn hedgerow. The pond was fed with drainage water from a ditch that ran along the margin of the maize field and by a ditch that flowed from the Spinney. Some drainage was also evident from the rape crop field via a small inflow into the main ditch running alongside the pond. Liquid manure was applied to the maize crop field, which on occasion was observed being over sprayed into the pond. This site was subject to pulses of high nitrate following field application of liquid manure via muck spreading and liquid manure applications. One sampling site was identified as the main body of the pond. The surface area of the pond was approx. 50 sq.m. *Triturus vulgaris* and *T cristatus* were found breeding in this pond; with over wintering *T.cristatus* larvae found in the pond during dip netting surveys.

Plate 1.1 Woodpond – Control site where residual nitrate concentrations were less than 3 mg/L NO^-_3-N for the duration of the testing period. Broadleaf woodland beyond with unimproved pasture in the foreground.

Plate 1.2 Fish Pond – Located on arable farmland with a sheep pasture beyond. Information from the farmer identified a field drain from the sheep pasture as the main water feed source for this pond.

Plate 1.3 Nature Reserve Pond - Located at the margin of two arable landuse types: a Winter Wheat (*T. aestivum*) crop and unimproved pasture.

Plate 1.4 Keyham Field Pond, incorporating Keyham Ditch (foreground). These sites were located at the junction of two Winter Wheat fields and at the bottom of slope.

Plate 1.5 Far Corner Field Pond – Picture taken on the 26th April 1994 with colleagues situated at back of pond at the main point of water inflow from a field drain. Outflow is at the front right of the photograph.

Plate 1.6 Far Corner Field Pond – Field drain inflow from surrounding field of Winter Wheat. This inflow was of a typical type observed at field drain fed ponds during the nitrate monitoring season.

Plate 1.7 Far Corner Field Pond – Photograph taken during late June 1994, with water levels substantially reduced. An extensive growth of Common *Persicaria sp.*, a 'bistort' related to *Polygonum amphibium*, formed an ideal habitat for emergent juvenile common frogs.

Plate 1.8 Spinney Field Pond - Photograph showing typical overhanging broadleaf trees and vegetation, boarded by a drainage ditch and a field drain inflow, indicated by the wooden post towards the back centre of the pond.

APPENDIX 3

Nitrate levels (mg/l NO_3-N) recorded for eleven sites during the Phase one (1994) sampling and monitoring programme. Sampling at each site was carried out on 46 separate occasions between 11 February 1994 and 3 February 1995 at each of the eleven sites.

Date	Far Corner Main	Far Corner Inlet	Far Corner Outlet	Spinney Pond Main	Date	Fish Pond Main	Fish Pond Inlet	Fish Pond Outlet	Wood Pond Main	Keyham Pond Main	Keyham Ditch Main	Nature Reserve Pond
16/2/94	29	34	30	49	02/11/94	56	48	51	1	16	18	9
23/2/94	26	27	26	39	18/2/94	69	79	66	1	20	16	6
03/02/94	29	28	35	50	25/2/94	60	61	50	0	8	14	1
03/09/94	31	34	32	53	03/04/94	81	88	75	0	18	21	10
16/3/94	28	28	34	40	03/11/94	51	71	38	<3	17	29	1
24/3/94	35	33	39	56	18/3/94	74	86	74	0	13	21	1
30/3/94	31	28	22	48	25/3/94	74	76	76	0	20	20	3
04/06/94	25	26	27	37	04/01/94	57	85	64	0	27	24	1
04/12/94	29	27	19	48	04/08/94	55	70	56	<3	16	15	6
20/4/94	25	31	21	38	15/4/94	52	65	53	<3	20	19	<3
26/4/94	41	32	23	39	22/4/94	59	90	67	<3	14	22	<3
05/10/94	30	8	5	30	29/4/94	54	D	65	<3	14	22	<3
17/5/94	3	24	1	23	05/06/94	35	D	39	<3	7	24	<3
25/5/94	0	15	0	17	13/5/94	41	D	46	<3	6	28	<3
06/02/94	0	18	0	23	22/5/94	26	57	28	<3	28	22	<3
06/08/94	0	16	4	21	27/5/94	48	d	38	<3	20	11	<3
17/6/94	2	d	2	8	06/03/94	43	d	37	<3	8	24	<3
07/01/94	<3	d	D	0	06/10/94	33	d	31	<3	4	d	<3
07/07/94	0	d	D	0	17/6/94	23	d	27	<3	<3	d	<3
14/7/94	D	d	D	<3	24/6/94	15	d	15	<3	<3	d	<3
18/7/94	D	d	D	<3	07/01/94	5	d	4	<3	d	d	<3
08/04/94	<3	d	D	<3	07/08/94	<3	d	<3	<3	d	d	0
08/11/94	D	d	D	1	15/7/94	<3	d	<3	<3	d	d	0
16/8/94	D	d	D	<3	22/7/94	<3	d	<3	<3	d	d	2
25/8/94	D	d	D	<3	29/7/94	<3	d	d	<3	d	d	1
31/8/94	D	d	D	0	08/04/94	<3	d	d	<3	d	d	1
09/07/94	D	d	D	0	08/11/94	<3	d	<3	<3	d	d	<3
14/9/94	D	d	D	<3	16/8/94	<3	d	d	<3	d	d	d
28/9/94	37	41	40	5	25/8/94	<3	d	d	<3	d	d	d
10/04/94	50	51	44	4	31/8/94	<3	d	d	<3	d	d	<3
13/10/94	40	41	40	<3	09/07/94	<3	d	d	<3	d	d	<3
20/10/94	23	34	35	<3	14/9/94	0	d	d	<3	d	d	1
11/02/94	40	41	28	4	23/9/94	28	d	d	<3	d	d	6
11/08/94	96	93	98	8	28/9/94	11	d	d	<3	d	d	<3
16/11/94	94	94	94	11	10/07/94	11	d	d	<3	d	d	<3
23/11/94	78	84	79	10	13/10/94	7	d	d	<3	d	d	<3

12/02/94	47	51	53	6	20/10/94	1	d	d	<3	d	d	1
12/08/94	84	77	82	3	28/10/94	1	d	d	<3	d	d	<3
16/12/94	83	83	87	9	11/04/94	18	d	d	<3	d	26	<3
21/12/94	64	62	65	9	11/11/94	98	93	94	<3	15	25	36
29/12/94	91	89	85	17	18/11/94	72	d	87	<3	11	d	0
01/05/95	54	43	54	12	25/11/94	45	d	58	<3	<3	d	<3
11-Nov	66	75	78	13	12/02/94	39	d	50	<3	1	13	<3
17/1/95	77	72	80	20	12/09/94	60	87	76	<3	15	13	7
26-1-95	32	33	38	26	15/12/94	56	d	48	<3	9	12	1
02/03/95	>90	67	76	45	22/12/94	50	d	34	<3	16	16	0
					29/12/94	94	d	43	<3	13	14	16
					01/06/95	74	d	48	0	16	14	7
					13/1/95	58	d	79	<3	14	20	1
					20/1/95	97	d	96	<3	13	17	29
					27/1/95	73	87	89	<3	13	16	20
d = DRY					02/03/95	82	96	88	<3	15	16	30

APPENDIX 4

Phase Two pond monitoring survey results showing the recorded (1996) concentrations of nitrate (mg/l NO_3-N) with mean values, recorded for 53 sites. Site name, water feed type ('Drain' = field drain; 'Run/rain' = runoff and rainfall; Run/leach = runoff and leaching; 'Stream' = fed by a connecting stream and 'Spring' = fed by underwater spring (determined from agricultural land use maps), presence and species of amphibians found (or evidence of; newt eggs in littoral foliage) at each site (s= smooth newts; c = crested newts; rt = common frog), numbers of frog spawns observed and the survey dates are displayed. **(*Rt = Ranatemporaria; **Tv = Triturus vulgaris; ***Tc = Triturus cristatus)**

Site Name	Grid Ref.	Species *Rt, **Tv, ***Tc	No. of frog spawns counted	Feed type	Recorded Nitrate concentration mg/l NO3-N on each sampling date										
					20.1.96	2.3.96	16.3.96	30.3.96	13.4.96	28.4.96	11.5.96	25.5.96	8.6.96	22.6.96	Mean
Little Stretton	SK 667002	*Rt, Tv*	*19*	*Drain*	22	25	28	28	54	6	44	47	35	42	34.3
Baggrave Hall	SK 699088	-	-	*Drain*		0	32	22	22	12	1	0	0		12.7
Baggrave Fish Pond	SK 698085	*Tv, Tc*	-	*Drain*		0	63	50	64	62	18	0	0		59
Brancliffe Farm	SK 703087	*Rt, Tv, Tc*	*5*	*Drain*		0	30	23	33	16	8	0	0		15.7
Quenby Moat	SK 701061	*Rt, Tv*	*7*	*Drain*		15	22	15	9	13	0	0	0		9.23
Prince of Wales Close	SK 695090	*Rt, Tv*	*26*	*Drain*		0	73	47	80	65	54	51	41	41	56.5
Gaulby Lane	SK 674024	*Rt,*	*7*	*Drain*	93	78	82	70	96	75	56	67	4		62.1
Village Farm 2	SK 684083	*Tv, Ts*	-	*Drain*		14	16	7	2	0	0	0	0		4.88
NR Fish Pond	SK 661051	-	-	*Drain*		45	70	59	63	53	44		18	2	44.25
Baggrave 2	SK 696086	*Rt, Tv*	*26*	*Drain*		49	79	55	55	28	7	0	0		34.56
NR Nature R. Pond	SK 661053	*Tv*	-	*Drain*		0	3	1	0	0	0	0	0		0.571
Silted Pond	SK 698072	*Tv, Tc*	-	*Drain*		139	124	111	126	86	62	94	22	35	88.78
Airport 1	SK 644014	-	-	*Drain*	37	26	14	12	10	11	0	2	0		12.44
Quenby Shaded Pond	SK 705066	*Rt,*	*28*	*Run/rain*		0	0	0	0	0	0	0	0		0
Baggrave 1	SK 696083	*Rt, Tv, Tc*	*85*	*Run/rain*		0	0	0	0	0	0	0	0		0

Inkerman Lodge 1	SK 705081	*Rt, Tv, Tc*	*31*	*Run/rain*		0	0	0	0	0	0	0	0		0
Square Spinney	SK 668051	*Rt*	*2*	*Run/rain*		0	0	0	0	0	0	0	0		0
Cold Newton	SK 711066	*Rt, Tv, Tc*	*32*	*Run/rain*		0	0	0	0	0	0	0	0		0
Tennis Pond	SK 689070	*Tv, Tc*	-	*Run/rain*		4	4	1	0	0	0	0	0		1.13
Small Pond	SK 682072	-	-	*Run/rain*		0	0	0	0	0	0	0	0		0
River Scence	SK 671009	*Rt, Tv*	-	*Run/rain*		0	0	0	0	0	0	0	0		0
Hungarton	SK 692076	*Rt, Tv*	*22*	*Run/rain*		0	0	0	0	0	0	0	0		0
Beeby	SK 664086	-	-	*Run/rain*		0	0	0	0	0	0	dry	Dry		0
Stretton Magna	SK 657005	*Tv, Tc*	-	*Run/rain*	14	0	0	0	0	0	0	0	0		1.56
South Lodge	SK 691083	*Rt, Tc*	*1*	*Run/rain*			51	51	49	47	24	13	13		31
South Croxton	SK 710097	-	-	*Run/rain*			0	0	0	0	0	0	0		0
Top Farm	SK 673001	-	-	*Run/rain*	9		7	5	0	9	0	0	0	0	3.75
HartField Lodge	SK 700082	*Rt, Tv, Tc*	2	*Run/rain*			0	0	0	0	0	0	0		0
Quenby New Pond	SK 704061	*Rt, Tv, Tc*	23	*Run/rain*	0		0	0	0	0	0	0	0		0
Airport Entrance	SK 650010	-	-	*Run/rain*	23		47	9	0	0	0	0	0		12.11
Hamilton	SK 643073	*Tv*	-	*Run/rain*	0		0	0	0	0	0	dry	Dry		0
Old Ingarsby	SK 684050	*Rt, Tv, Tc*	8	*Run/leach*	23		30	25	21	1	0	0	0		12.5
Houghton On Hill	SK 673034	*Rt, Tv, Tc*	19	*Run/leach*	36		32	21	63	1	0	0	0		19.13
Quenby Lodge	SK 708065	*Rt, Tv*	1	*Spring*	0		0	0	0	0	0	0	0		0
Hall Spinney	SK 704089	*Rt*	45	*Stream*			15	4	0	0	0	0	0		2.71
Observatory	SK 686079	*Tv*	-	*Stream*	34		32	25	40	24	13	5	0		21.63
Quenby Fish Lake	SK 701068	-	-	*Ditch*	1		1	0	0	0	0	0	0		0.25
Quenby Fish Pond	SK 694062	*Tv, Tc*	-	*Ditch*	0		0	0	0	0	0	0	0		0
136	SK 700077	*Tv, Tc*	-	*Ditch*			1	3	0	0	0	0	0		0.571
Village Farm 3	SK 683082	*Tv, Tc*	-	*Ditch*	22		23	19	23	10	1	0	0		12.25
Airport 2	SK 644108	*Rt, Tv, Tc*	4	*Run/rain*	0		0	0	0	0	0	0	0		0

Great Stretton	SK 655005	-	-	*Run/rain*		3	2	0	0	1	0	0	0		0.75
Oadby	SK 632019	*Rt, Tv*	*10*	*Run/rain*		0	0	0	0	0	0	0	0		0.22
White's Barn	SK 708074	*Rt, Tv, Tc*	*8*	*Run/rain*		0	0	0	0	0	0	0	0		0
Watson's Spinney	SK 688085	-	-	*Run/rain*		4	4	0	0	0	dry	dry	Dry		1.6
Corn Close	SK 655025	*Rt, Tv, Tc*	*50*	*Run/rain*		1	1	0	0	0	0	0	0		0.22
Village Farm 1	SK 695086	*Tv, Tc*	-	*Run/rain*		0	0	0	0	0	0	0	0		0
NR Woodpond	SK 665053	*Rt, Tv*	*19*	*Run/rain*		0	1	0	0	0	0	0	0		0.13
Top Farm 1	SK 672999	-	-	*Run/rain*		0	0	0	0	0	0	0	0		0
Inkerman Lodge 2	SK 706081	*Tc*	-	*Run/rain*			0	0	0	0	0	0	0		0
Hungarton Lane	SK 679076	*Rt, Tv, Tc*	*8*	*Run/rain*		0	1	0	0	0	0	0	0		0.13

APPENDIX 4

Measured concentrations of residual nitrate (mg/l NO_3^--N) recorded in the second flow-through test measured using the Reflectoquant portable nitrate meter 'RQ-Flex'. Values in brackets are daily measured concentrations expressed as a percentage of the values recorded on Day 0 for each fertiliser concentration.

Day Number	Measured concentrations mg/l NO_3^-N				Measured concentration as a % of the nominal concentrations with % of Day 0 values in brackets.			
	Control	25	50	100	Control	25	50	100
0	-	19	61	109	-	76 (-)	122 (-)	109 (-)
1	-	18	65	108	-	72 (95)	130 (107)	108 (99)
2	-	16	66	104	-	64 (84)	132 (108)	104 (95)
3	-	19	64	109	-	76 (100)	128 (105)	109 (100)
4	-	26	63	125	-	104 (137)	126 (103)	125 (115)
5	-	29	54	121	-	116 (153)	108 (89)	121 (111)
7	-	34	50	119	-	136 (179)	100 (100)	119 (109)
10	-	37	39	103	-	148 (195)	78 (64)	103 (94)
18	-	31	49	104	-	124 (163)	98 (80)	104 (95)
26	-	34	56	110	-	136 (179)	112 (92)	110 (101)
28	-	28	52	114	-	112 (147)	104 (85)	114 (105)
35	-	26	59	126	-	104 (137)	118 (97)	126 (116)
43	4	21	58	117	n/a	84 (111)	116 (95)	117 (107)
45	-	18	74	139	-	72 (95)	148 (121)	139 (128)
51	-	19	54	101	-	76 (100)	108 (89)	101 (93)
59	3	24	53	98	n/a	96 (126)	106 (87)	98 (90)
68	-	21	70	84	-	84 (111)	140 (115)	84 (77)
76	-	23	62	82	-	92 (121)	124 (102)	82 (75)
79	-	33	85	81	-	132 (174)	170 (139)	81 (74)
82	6	31	81	65	n/a	124 (163)	162 (133)	65 (60)
86	-	27	65	89	-	108 (142)	114 (107)	89 (82)
90	-	17	57	95	-	68 (89)	114 (93)	95 (87)
93	-	21	58	97	-	84 (111)	136 (95)	97 (89)
96	-	26	56	99	-	104 (137)	112 (92)	99 (91)

Average	**0.542**	**24.9**	**60.5**	**104.1**